INFLAMMATORY PATHWAYS RELATED TO SLEEP-DISORDERED BREATHING AND PERIODONTAL STATUS

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A thesis submitted to the faculty at the University of North Carolina in partial fulfillment of the requirements for the degree of Master of Science in the Adams School of Dentistry (Periodontology).

Chapel Hill
2021

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ABSTRACT

John Bruce: Inflammatory Pathways Related to Sleep-Disordered Breathing and Periodontal Status
(Under the direction of Antonio Moretti)

Aims: (1) Investigate inflammatory aspects of periodontal disease (PD) and obstructive sleep apnea (OSA), in isolation or co-exist in same individuals, (2) Investigate how the profile of salivary cytokines, associated with periodontal disease, is altered by co-existing obstructive sleep apnea.

Methods and Materials: Participants were recruited at the University of North Carolina Adams School of Dentistry. Each participant underwent a thorough periodontal examination, saliva sample collection, and a two night home sleep study from which the apnea-hypopnea index (AHI) was estimated. Saliva samples were processed for fourteen inflammatory marker profiles of 81 participants. Participants were grouped according to clinical periodontal inflammation. Bleeding on probing (BOP) on ≥20% of sites indicated a high level; BOP on less than 20% of sites, a low level. Analyses of biomarker data were completed using regression to control for demographic, behavior and medical factors.

Results: IL-6 (p-value = 0.02) and IL-10 (p-value = 0.02) were 119% and 157% higher, respectfully, in individuals with BOP≥20% as compared to individuals with BOP<20%. Monocyte Chemoattractant Protein 1 (MCP-1) increased exponentially with the AHI (p-value = 0.01) and there was a trend for IL-6 and IL-10 to increase with AHI also (p-values = 0.08). In no case did the effect of OSA on the biomarkers differ for the two groups of participants.

Conclusions: Few salivary biomarkers of inflammation were elevated by either active PD or OSA in the present study. Concentrations of the two biomarkers elevated in PD, IL-6 and IL-10, also tended to increase with the severity of sleep-disordered breathing similarly for individuals with or without PD. No

evidence was found to suggest that pathways of inflammation associated with the two diseases interact synergistically.

ACKNOWLEDGEMENTS

First, I would like to thank and acknowledge Dr. Antonio Moretti for his constant support, drive, patience, understanding, and guidance through the process of this project. Thank you as well for helping me develop during my time in this residency program. Without your help there is no way I would have been able to complete this project. I would also like to thank my other committee members Dr. Gregory Essick and Dr. Silvana Barros for their supportive and integral roles overseeing this project. Your insight, mastery level knowledge, and thoughtfulness proved invaluable throughout the last three years. Further, outside of my direct committee I would like to thank Russell Levy with his assistance in overseeing the lab work of this project, and ensuring that work was sound and completed in a proper manner. Next, I would like to thank Kevin Moss for the generous donation of his personal time to see through this project and to dramatically impact the level of statistical evaluation that we were able to complete for this project. Finally, of course I would like to thank my other faculty, staff, and co-residents for their influence and support throughout my three years at UNC ASOD.

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LIST OF ABBREVIATIONS AND SYMBOLS

AASM – American Academy of Sleep Medicine

AHI – Apnea-hypopnea index

ANOVA – Analysis of Variance

BMI – Body Mass Index

BOP – Bleeding on probing

CAL – Clinical attachment loss

CAP – Community acquired pneumonia

CD4 – Cluster of Differentiation 4

CD8 – Cluster of Differentiation 8

CPAP – Continuous Positive Airway Pressure

CVD – Cardiovascular Disease

ELISA – Enzyme-Linked Immunosorbent Assay

GCF – Gingival crevicular fluid

HIF-1α – Hypoxia-Inducible Factor 1α

IgG - Immunoglobulin G

IL-10 – Interleukin 10

IL-13 – Interleukin 13

IL-17 – Interleukin 17

IL-18 – Interleukin 18

IL-1α – Interleukin 1α

IL-1ß – Interleukin 1ß

IL-2 – Interleukin 2

IL-22 – Interleukin 22

IL-3 – Interleukin 3

IL-4 – Interleukin 4

IL-5 – Interleukin 5

IL-6 – Interleukin 6

IL-8 – Interleukin 8

INF-ϒ – Interferon ϒ

LDL – Low Density Lipids

LPS – Lipopolysaccharide

MCP-1 – Monocyte Chemoattractant Protein - 1

NO – Nitric Oxide

OSA – Obstructive Sleep Apnea

PD - Periodontal Disease

PMNs – Polymorphonuclear Leukocytes

REM – Rapid Eye Movement

ROS – Reactive Oxygen Species

TBARS – Thiobarbituric Acid Reactive Substances

TGFß – Transforming Growth Factor ß

Th17 – T-Helper 17

TNFα – Tumor Necrosis Factor α-

CHAPTER 1: PATHOPHYSIOLOGY AND INFLAMMATORY PATHWAYS OF PERIODONTAL DISEASE AND OBSTRUCTIVE SLEEP APNEA

PATHOGENESIS OF PERIODONTAL DISEASE

Periodontitis is a host-mediated infectious disease of inflammatory nature, usually associated with bacterial biofilm and calculus, characterized by loss of supporting tissues (e.g., alveolar bone and connective tissue) of teeth, progressive attachment and bone loss ultimately resulting in pocket formation and, in many situations, gingival recession.[1] In large part periodontitis is a disease in which altered wound healing properties are found.[2] In physiological conditions the periodontal complex of the mouth is characterized by a constant remodeling of the area resulting in a stable supporting structure or extracellular matrix. While this homeostasis always has a combination of inflammatory reducing and pro-inflammatory effectors, there is a maintained, delicate balance, which allows for the correct level of alveolar bone resorption, to permit for proper remodeling. When host response is altered or there is a change in microbial composition, this can ultimately lead to a change in homeostasis resulting in a change to degradation in the affected areas as more inflammatory producing markers and cells are released and recruited to the area.[3] Furthermore, oxidative stress resulting from different microbial interactions resulting in excess reactive oxygen species or deficient antioxidants can also contribute to degradation of the periodontal complex.[4] When this shift occurs in an extreme or chronic nature, we eventually begin to see signs of periodontitis, or loss of alveolar bone and supporting connective tissue

structure around teeth. This loss of supporting structure has typically been measured in terms of clinical attachment loss, periodontal probing depth, mobility, and radiographic bone loss.

Periodontitis is often divided into four different stages: Initial lesion, early lesion, established lesion, and ultimately the advanced lesion where we begin to see alveolar bone loss.[5] The initial lesion is often clinically non-detectable but it can be identified histologically in response to bacterial biofilm. Specifically, in this stage, endothelial cells are impacted by metabolic products and ultimately secrete cytokines in order to, among other events, cause vasodilation and recruit neutrophils, macrophages, and leukocytes to the area triggering the beginning of the inflammatory response.[6] The early lesion is identified by an influx of neutrophils to connective tissue and the presence of macrophages and lymphocytes, among other cells. At this point signs of inflammation are observed both histologically and clinically in the form of gingivitis and increased gingival crevicular fluid is observed.[6] Gingivitis is said to affect approximately 75% of adults in the United States and is characterized clinically by redness, swelling, and bleeding of the gingiva.[3] In the established lesion macrophages, plasma cells, T and B lymphocytes are primarily present.[6] Blood flow will be decreased and increased collagenase activity can be seen as a more moderate to severe form of gingivitis appears clinically.[6] Finally, in the advanced lesion there is a transition to clinical periodontitis as histologically irreversible attachment and alveolar bone loss are observed. Advanced forms of periodontitis are prevalent in the population affecting approximately 30% of the individuals in a moderate form and 10% in the most advanced form.[3]

Typically, periodontitis is diagnosed based on several different parameters. Probing depth and clinical attachment loss (CAL) can be measured either buccal or lingual or interproximally for every tooth in the mouth. In a healthy and intact periodontium, there should be no loss of

attachment, or CAL, and pocket probing depths should be 3mm or less.[3] Periodontal pocketing in patients with 4mm or more can be diagnosed as periodontitis, if combined with CAL, and over 6mm with more advanced degrees of the disease.[3] In 2018, the American Academy of Periodontology, together with the European Federation of Periodontology, released a new periodontal classification system for staging and grading patients' disease progression. This newest classification is based primarily on interdental CAL, radiographic bone loss percentage, and tooth loss related to periodontal disease.[7] Stage I describes CAL of 1-2mm and radiographic loss of less than 15% with no tooth loss.[7] Stage II describes CAL of 3-4mm and radiographic loss of 15-33% with no tooth loss.[7] Stage III is defined by 5mm or more of CAL, radiographic loss of bone past the middle third of the tooth and loss of 4 or less teeth.[7] Finally, stage IV is the most advanced stage of over 5mm of CAL, radiographic loss extending to the middle third of the tooth or more, and loss of 5 or more teeth.[7] Many other complexity factors are also considered, when determining the staging, such as maximum probing depth, vertical bone loss, furcation involvement, ridge defects, etc.[7] Grading is used to define the rate of progression of the disease. This area of the classification is largely based on direct evidence of progression and percentage bone loss by age.[7] Other risk factors are accounted for such as smoking and diabetes and the grades are defined as A, B, or C, with A being considered a slow rate, and C being considered a rapid rate of progression.[7]

Periodontitis has many distinct effects in the human body. As mentioned previously, the presence of bacteria has the capability to produce tissue destruction and, therefore, also activate host defense causing initial cells such as lymphocytes, macrophages, and polymorphonuclear leukocytes to be recruited to the area.[3] While there are known to be hundreds of bacteria in

dental microbial biofilm, several have been identified specifically as periodontal pathogens.[3] Specifically, Socransky defined several complexes of bacteria commonly seen in disease with an emphasis on the orange complex, and more importantly the red complex of bacteria.[8] The described red complex included bacteria *Porphyromas gingivalis*, *Treponema denticola*, and *Bacteroides forsythus (now Tannerella forsythia)*. Further, while not part of the red complex, it was also identified *Aggregatibacter actinomycetemcomitans* was commonly seen in very severe cases.[8] The cells respond to these bacteria and their bacterial markers such as lipopolysaccharide (LPS) and eventually begin to synthesize and secrete different proinflammatory molecules such as cytokines, chemokines, prostaglandins, and more.[3] These markers play a role in the breakdown of periodontal tissue and eventually cause reactive oxygen species which help elevate collagenase in the gingival tissue.[3] A combination of these catabolic activates and metalloproteinases will eventually lead to tissue destruction and ultimately the attachment loss measured in diagnosis of periodontal disease.[3]

Systemically, periodontitis has been shown to influence many different systems and processes in the body. One example of systemic interaction is a correlation between periodontal disease and cardiovascular disease (CVD). One study demonstrated that 91% of CVD patients had been diagnosed with either moderate or severe periodontitis.[9] It has also been identified that many of the same oral bacteria exist in carotid, coronary, and other atherosclerotic plaques.[3] The bacteria, specifically *P. gingivalis*, are thought to invade the arterial walls of patients through and interaction with the endothelial surface and as a response to the infection and damage to endothelial cells, a release of inflammatory mediators result in an atherogenic effect.[3] This in turn is known to effect several functions including nitric oxide (NO) availability.[3] As a

consequence of less bioavailability of NO there is thought to be a decreased oxidative inactivation by reactive oxygen species (ROS), which ends in a systemic increase in inflammatory cytokines.[3] Additionally, NO has been shown to be beneficial in host defense to specifically *P. gingivalis* with increased levels being shown to reduce inflammatory cell infiltrate in rats.[10] This decrease in NO bioavailability not only may cause an increase in inflammatory cytokines but also affect the hosts ability to control *P. gingivalis.*[9] Additionally, it has been shown that periodontitis is directly affected with the ability of low density lipoprotein (LDL) to activate macrophages resulting in binding of LDL to foam cells, further increasing the risk of CVD.[11] Several other interactions are also being used to confirm the association of periodontal disease and cardiovascular disease.

Another systemic interaction can be seen with diabetes mellitus where there is a higher prevalence of periodontal disease compared to heathy controls.[12] There is evidence to suggest that periodontal pathogens were seen in higher number in diabetic patients and that they may further aggravate microvascular complications such as retinopathy coronary artery disease, and peripheral vascular disease.[3] It is also established that there is a significant bidirectional relationship between the two diseases showing that periodontal disease may worsen with diabetes and that periodontal disease may cause insulin resistance.[3] Adipose tissue is shown to be the source of several proinflammatory cytokines which ultimately contribute to insulin resistance, and upregulation of specific receptors which may increase chance for a more rapid and severe response to a hyperinflammatory situation, such as periodontitis.[3] The induced hyperinflammatory state caused by the adipocytes may be the link between type 2 diabetes, obesity, and periodontal disease.[3] In addition, it has also been shown that effective control of periodontal disease may help glycemic control in diabetic patients.[13]

Finally, periodontal disease has been seen to be associated with osteoporosis and also estrogen deficiency.[3] It is commonly known that chronic inflammation will ultimately lead to osteoclastic resorption through activation of T-lymphocytes and up regulation of osteoclasts.[3] Estrogen, which has important roles in maintaining bone density in females, has a regulatory effect on cytokines for bone metabolism and host response. It is thought that having both estrogen deficiency and periodontal disease may be enough to induce osteoporosis.[3]

PATHOGENESIS OF OBSTRUCTIVE SLEEP APNEA

Obstructive sleep apnea (OSA), on the other hand, is a disorder characterized by episodes of complete or partial collapse of the upper airway, which results in either a cessation or reduction of airflow during sleep.[14] While sometimes partial collapse of the airway is considered normal, OSA is defined by obstructions of airway that last a minimum of 10 seconds with often a reduction in blood oxygen saturation.[15] The severity of OSA is commonly measured on a continuous scale by the apnea-hypopnea index (AHI) or the number per hour of sleep of complete (apneas) and partial (hypopneas) obstructions to airflow that meet well-defined criteria. A ≥90% reduction in airway defines an apnea. A partial obstruction to airway (≥30% but <90% reduction) meets criteria for a hypopnea when it is associated with an arousal from sleep or a desaturation in blood oxygen of ≥3 or 4%.[16,17] AHI is considered normal or subclinical when there are less than 5 events an hour, mild from 5-15 events, moderate at 15-30 events, and severe at over 30 events per hour.[16] Oxygen desaturation is considered significant at 3%, identifying a more mild event, while events causing a desaturation over 4% identify a more severe obstruction.[17] Further, another important measure obtained from sleep testing is the cumulative percentage of time for which

blood oxygen saturations is below 90%, otherwise known as CT90. Studies have shown there is a correlation between the polysomnographic AHI measure and CT90.[18]

OSA symptoms include snoring and disrupted and non-refreshing sleep, which oftentimes manifest as daytime sleepiness or other daytime neurobehavioral problems.[19] Neurobehavioral issues include functional impairment such as increased risk for accidents while driving, and impairment in memory, vigilance, psychomotor performance, and constructional abilities, among other issues.[20] Further, it has been shown, by several studies, that decision making performance and prediction of performance are closely linked to minimal oxygen saturation during a night's sleep.[21] While many individuals can have mild asymptomatic forms of sleep apnea, in moderate to severe forms complications of great concern can develop including metabolic disease and weight gain, unproductiveness, cardiovascular disease, systemic and pulmonary hypertension, and a higher risk of stroke, to name a few.[22]

More specifically, it has been shown that individuals with OSA have an increased risk of cardiovascular mortality, morbidity, and sudden death during the night time.[22] Most of the sleep-related cardiac effects are attributed to nocturnal sympathetic activation, which disrupts the normal resting protective effect of non-rapid eye movement (REM) sleep on the cardiac system.[22] Constant interruption of sleep arouses the hypothalamic-pituitary-adrenal axis, which activates the sympathetic adreno-medullary system raising nocturnal blood pressure, potentially inducing left ventricular hypertrophy, and early left ventricular diastolic dysfunction.[23] The end result includes endothelial damage and dysfunction, systemic inflammation, increased oxidative stress, and increased daytime hypertension.[22] Ultimately there can be a higher mortality associated with sleep disruption from obstructions to airflow.[22]

Further, metabolic disease has also been linked to OSA. A recent study showed higher risk of hyperglycemia and hyperinsulinemia in individuals with less than 6 hours of sleep and OSA regardless of body mass index (BMI).[24] In addition, recent studies have shown that insulin and insulin resistance levels correlate with the severity of sleep apnea in a positive relationship.[24]

The gold standard treatment of OSA is continuous positive airway pressure (CPAP) to maintain patency of the airway.[15] Recent studies have demonstrated the importance of adherence to CPAP therapy. Without use of CPAP for more than 6 hours per night, patients often will still have symptoms and suffer the medical complications of moderate to severe sleep apnea, attesting further to the potential negative effect that sleep apnea may have systemically.[16] While CPAP eliminates many symptoms patients report, there is still a lack of long-term evidence supporting elimination of cardiac issues associated with OSA. Further, many patients find use of CPAP intolerable and it is known for having poor compliance among patients.[15]

While the disease process of OSA is quite complex, many believe it is related to the intermittent hypoxia, intra-thoracic pressure changes, and as mentioned above, sympathetic nervous system activation.[15] At its core, the repetitive fluctuations in oxygen, pressure and sympathetic drive during sleep have been shown to trigger oxidative stress mechanisms in cells.[25] A few examples of the cellular effects, leading to systemic changes, have been found thus far. During the hypoxic phases, cells begin adapting to low oxygen concentrations and when reoxygenation occurs this is thought to cause production of reactive oxygen species (ROS).[15] ROS is a term used to describe these different molecules and free radicals derived from molecular oxygen.[26] In its ground state, oxygen has a low reactivity as it is stable with two electrons in the outer shell, have the same spin, and are only able to react with one electron at a time.[26]

Superoxide anion is a product of electron reduction and the precursor for most ROS as it can ultimately form hydroxyl radicals and react with other radicals such as nitric oxide.[26] Under hypoxic conditions small amounts of nitric oxide may bind and inhibit cytochrome oxidase and increase the electron reduction of carriers at the terminal oxidase making it more likely to form superoxide anion.[26] ROS are than able to signal downstream, effecting potassium channels which cause acute changes as a response to altered oxygen levels.[15] Furthermore, there are several oxidant-mediated vascular smooth muscle transductions that cause promotion of systemic hypertension through the carotid body and eventually activation of the renin-angiotensin II axis.[15]

Other effects of ROS production, due to hypoxia, include the ability of these oxide radicals to be capable of oxidizing lipids, proteins, and DNA.[15] ROS also alter different biological functions including signal transduction of oxygen identifying mechanisms and cell growth.[15] The specific production of ROS in OSA patients plays a role in inflammation of these patients and potentially also the increased sympathetic tone and elevated catecholamine levels.[15] There is significant evidence showing several pathways of oxidative stress related to OSA leading to inflammation.[15] One pathway is related to release of superoxide from polymorphonuclear neutrophils (PMNs) which was shown to be decreased with CPAP treatment.[15] Another pathway is related to increased expression of CD15 and CD11c adhesion molecules leading to higher monocyte attachment to endothelial cells.[15] This upregulation was shown to be associated with hypoxia and again the effect was reduced with treatment of CPAP.[15]

There are many factors that influence oxidative stress such as diet, environment, and genetics. Measuring markers for oxidative stress can be challenging as one often needs large

sample sizes, proper storage, and very sensitive techniques to access them. One of the ways researchers report being able to measure oxidative stress is by looking at lipid peroxidation profiles, which tend to be higher in untreated OSA patients. Another way is by looking at cellular antioxidant defense mechanisms, which tend to be lower in patients with oxidative stress.

OSA and oxidative stress are known to impact gut microbiota and diversify the communities of bacteria in mice.[27] These changes may impact the physiological functions of the body and lead to lasting increases in endotoxin levels.[27] Another example of OSA's impact on host defense is the increased susceptibility to community acquired pneumonia (CAP).[28] It was shown that OSA triples the risk of CAP and that this is also related to the severity of OSA.[28] Again, it is thought that part of the reason for this increased susceptibility is due from the alteration of the microbiota composition and diversity of the intestines causing a physiological dysregulation.[28] This is a current area of interest in modern research and is still being widely explored.

INFLAMMATORY RESPONSE

Cytokines are low molecule weight proteins that help initiate inflammation and regulate the level response in the area of concern.[6] Cytokines are produced by local resident cells and by phagocytes in both the acute and chronic phases of inflammation, as well as by immune cells in more advanced lesions.[6] When invading microorganisms are recognized, cytokines are triggered by an innate response of the cells.[6] As an example to be discussed in depth further, cytokines such as IL-1ß and IL-6 appear early in the pathogenesis pathway and are characterized by their ability to signal further inflammatory cell migration and osteoclastogenesis.[6]

T-helper lymphocytes have different immunoglobulin molecules present which functions as receptors for antigens in cell-mediated immunity. When bound, this receptor is able to recognize

fragments of pathogens and ultimately ends in activation of macrophages, B cell, and other T-cells.[6] In turn, the recruited cells and T-helper cells will result in secretion of different cytokines, chemokines, and other mediating factors to inspire either a pro-inflammatory or anti-inflammatory effect. Generally there are two accepted classifications of T-cells based on their cell surface expression of "cluster of differentiation" 4 or 8, otherwise known as CD4 or CD8.[6] While both cell types have a meaningful effect, generally CD4 is focused on for its known effect in inflammatory processes. CD8 cells are immune effector cells and also secrete cytokines, however.[6] Further, CD4 cells are normally broken down into two categories, and more recently two more have been accepted. Th1 are typically pro-inflammatory as they often produce cytokines such as INF-ϒ and IL-2, among others.[6] This subset will ultimately enhance the cell-mediated responses locally and is a known contributor for initiation of osteoclastogenesis.[6] Th2 subsets are generally recognized as anti-inflammatory and it is generally accepted that they suppress cell-mediated responses.[6] This subset is known to secrete many cytokines such as IL-4, IL-5, IL-6, IL-10, IL-13.[6] Both subsets are known to secrete IL-3, TNF-α, and macrophage stimulating factors in certain circumstances, as well as impact the behavior in secretion of B-cells in the affected area.[6] As previously mentioned, more recently Th17 is a newly recognized subset which is now known to produce IL-17 and IL-22 acting to effect or suppress cells depending on the local environment.[6] Th17 has a known effect in chronic periodontitis sites and is activity produced in periodontal lesions. Finally, T-regulatory cells are recognized as protective and as known producers of IL-10 and TGFß.

As far as measuring cytokines there are several methods involving blood or plasma samples, and also saliva and gingival crevicular fluid. Salivary and GCF samples have the advantage of being

easily collected from patients and have been shown to have the ability to show local and systemic inflammatory markers.[29] Initial studies involving GCF were concerned with if it was a product of inflammation or health. It was eventually discovered that GCF was produced likely as part of a defense mechanism and that the end capillaries and soft tissue in the sulcus was permeable and allowed proteins to pass through connective tissues.[30] Saliva has several different functions in both bacterial defense, one's innate immune system, digestion, and buffering. This being said, with recent advances in protein analysis saliva has been shown to contain biological markers which is activity being explored to potentially diagnose several disease and conditions.[31]

While there are many markers that are currently being explored for their roles in inflammation, there are many that are currently recognized for their extensive roles in both periodontal disease and OSA. In addition, there are many markers that researchers have been able to successfully isolate and identify in these conditions. For the next part of this literature review, we will discuss some specific cytokines that have been shown to be associated and/or correlated with either the presence, or absence of periodontitis and OSA, and ultimately how these cytokines may be used to help identify a potential correlation between these two conditions.

CYTOKINES OF INTEREST

IFN-ϒ is a pleiotropic cytokine which has several roles in both pro-inflammatory and the immune systems.[32] More specifically IFN-ϒ induces cytokine production through positive feedbacks loops and upregulates major histocompatibility complex (MHC) antigens on different cell membranes.[32] IFN-ϒ pro-inflammatory functions stem from the fact that is influences Th cell phenotype by inhibiting formation of the TH2 and TH17 linages, while stimulates TH1

development.[32] It is known that this pro-inflammatory effect is likely to have a role in chronic inflammation.[33] It was discovered in mice that IFN-Ɣ, among other cytokine profiles, help to stimulate IL-6 and eventually influencing myeloid-derived suppressor cells (MDSCs).[33] IFN-Ɣ effect on MDSCs is to cause these cells to suppress less, enabling inflammation to occur without an end point.[33] This being said, studies have explored the role of IFN-Ɣ in OSA as it is a source of chronic inflammation. IFN-Ɣ was shown to be consistently higher in the connective tissue of OSA patients.[34] Further, importantly for periodontitis, it is an activator of macrophage effector functions including synthesis, class switching, and B cell secretion. IFN-Ɣ acts on macrophages to positively regulate IL-12 production and induce other pro-inflammatory cytokines such as IL-1ß and TNFα to be secreted.[35] Many studies have proven this association between IFN-Ɣ and periodontal disease. One study showed that IFN-Ɣ was seen in higher levels in periodontal disease and was correlated with bone resorption.[36] Finally, in another study, IFN-Ɣ has also shown to be increased in what was formerly referred to as aggressive periodontitis, and less significantly with chronic periodontitis.[35]

IL-4 is considered an anti-inflammatory cytokine. IL-4 is an important cytokine for growth and proliferation of B lymphocytes and is mostly secreted by TH2 cells, and also has an effect to help amplify the TH2 immune response.[37,38] IL-4 also, in part, leads to the inhibition of other known pro-inflammatory cytokines such as TNFα, IL-6, IL-8, and IL-1ß through direct inhibition, and through production of IL-10.[38] In addition to its action reducing pro-inflammatory output, IL-4 also has been shown to inhibit reactive oxygen and nitrogen series formation, and prostaglandins, which all influence local inflammatory effects.[38] Further, another reason it is considered anti-inflammatory is due to its ability to cause macrophage apoptosis.[38] Additionally,

IL-4 also has an effect on osteoprotegin (OPG) causing downregulation of Receptor activator of nuclear factor kappa-B ligand (RANKL) and reduction of bone resorption.[38] This being said there have been several studies recognizing IL-4's function in periodontal health. Specifically, one study showed that there was an inverse relationship reporting in gingival crevicular fluid (GCF) IL-4 levels and the patient's periodontal status, showing lack of IL-4 at inflamed sites.[38] The conclusion of this study postulated that lack of IL-4 is associated with periodontal destruction.[38] With relation to OSA, the role of IL-4 is slightly different. It has been shown that IL-4 has a role in producing a protein called periostin which is typically known for its role in bone formation in response to parathyroid hormone, and is being investigated as a potential marker for periodontal disease.[37] However, production of this protein in nasal and lung tissue has been shown to potentially increase pro-inflammatory responses as it also amplifies the Th2 immune responses in allergic airway disease.[37] This being said there is a thought that IL-4 and its production of periostin could have a role in persistent airway inflammation.

IL-10 is also largely considered an anti-inflammatory marker. T-regulatory cells with protective functions are known producers of IL-10.[6] IL-10, among other functions is associated with suppressing the expression of IFN-γ, TNFα, IL-1ß, IL-12 and IL-17 as well as downregulating matrix metalloproteinases (MMPs), RANKL, and myeloid-derived suppressor cells (MDSCs).[6,24,33] With regard to periodontal disease it has been shown that patients with defective IL-10 were not able to properly manage bacteria resulting in bone loss and more severe disease.[36] One study showed IL-10 as a promising biomarker for identification of individuals without OSA.[19] Another study also showed that low IL-10 is associated with obesity, insulin resistance, and was correlated with higher Apnea Hypopnea Index (AHI) levels.[24] Further, it was shown that normal levels of

plasma IL-10 levels were restored following surgical procedures which helped resolve sleep apnea, inferring that hypoxia may lead to interruption of its synthesis.

IL-13 is another cytokine with anti-inflammatory actions. IL-13 is a known influencer of human monocyte and macrophage functions by upregulation of MHC and integrins.[6] Further, it is also noted that IL-13 helps to inhibit the functions of many pro-inflammatory markers such as IL-1a, IL-1b, IL-6, IL-8, and TNFα. Additionally, it has been shown to upregulate production of IL-4, which shared many of these same functions.[6] IL-13 has a significant role in periodontitis due to its regulatory actions. More specifically IL-13 is considered fibrogenic as it causes stimulation of gingival fibroblasts by causing production of TGFß and has been shown to cause a significant decrease in MMP-1 production.[39] As for OSA, IL-13 has a similar contributory effect as IL-4 by influencing the production of periostin, which was previously discussed.[37]

IL-8 is commonly known to work as a pro-inflammatory agent. IL-8 is often produced by fibroblasts and has specific roles as a chemo-attractor for neutrophils, monocytes, and lymphocytes.[40] In normal conditions, IL-8 will cause neutrophil extravasation to help defend against local bacteria; however, in excess, IL-8 is tied to local tissue destruction through T-cell activation.[40] In terms of periodontal disease IL-8 is often seen in high concentration in initial lesions, where there is only subclinical inflammation present.[6] Further, IL-8 is also known to have a direct effect on osteoclast differentiation and can cause secretion of MMP-8 and MMP-9.[6] On the other hand IL-8 has been seen to be positively correlated with AHI and is known to influence angiogenesis in atherosclerotic tissue and its release has been tied to hypoxic events.[41] Further, another study also showed that there is an increase in IL-8 when hypoxic stress is higher, even in healthy individuals.[42]

IL-18 is a known pro-inflammatory cytokine which was initially thought to be a signaling factor for IFN-ϒ, and can also work synergistically with IL-12.[43,35] Interestingly enough, IL-18 is unique in that it can induce either Th1 or Th2 differentiation and is expressed in monocytes and macrophages.[43] Through this interaction it has been shown that IL-18 ultimately leads to the production of MMP-9 and also IL-1ß.[43] In terms of its role in periodontitis, several studies have showed a distant connection. IL-18 is readily available in oral epithelial cells and is associated with the presence of *P. gingivalis* and some research is showing it may be a marker for detection of gram-positive bacteria.[43,35] Studies have also shown that in GCF, and biopsies of gingiva, greater local levels of IL-18 were associated with greater pocket probing depths.[35] Further, it has been shown that there is a reduction in IL-18 following scaling and root planing in individuals.[43] In terms of sleep apnea, again it was shown that IL-18 was seen at increased levels in individuals with OSA, when correcting for patient obesity.[44] Further, IL-18 was seen as a contributing factor toward atherosclerosis and increased intima-media thickness, which is linked to OSA.[44] Most importantly it was also noted that production of other pro-inflammatory cytokines such as IL-6, IL-1ß, and TNFα, obesity, and hypoxia all were involved in elevating IL-18 in OSA patients.[44]

TNFα is seen as a main mediator of pro-inflammation and has been widely explored in research since it's discovery. This is a cytokine that has many functions, some of which include cell migration for tissue destruction and adhesion of neutrophils to vessel walls.[6] TNFα has a significant effect on monocytes, macrophages, and fibroblasts and works synergistically with both IL-1α and IL-1ß.[40] In excess, TNFα also activates pathways which result in connective tissue destruction through secretion of collagenase and MMPs.[6,40] Further, it causes resorption of cartilage and bone through secretion of RANKL and activation of osteoclasts, and through

positive feedback with other proinflammatory markers such as IL-1B specifically.[6,40] This being said TNFα is a known enhancer of bone resorption due to these effects and may also be a sign of early inflammation.[40] Interestingly, TNFα is highly expressed from adipose tissue and have been shown by many studies to be drastically increased in obese patients.[3,24,45 ,46] Further, has also been seen to be leading factor in development of insulin resistance in patients by impairing glucose metabolism.[3] In terms of periodontal disease the role of TNFα has been long established by many studies due to its influence on tissue destruction. In one study it was shown that in combination with IL-13, TNFα showed a two-fold increase in MMP-1 production, a factor that is essential in gingival turnover.[39] Also, it is thought that the hyper-inflammatory state it causes may be a risk factor for development of periodontal disease.[3] Further, another study showed the high correlation of IL-1ß and TNFα specifically in patients with periodontal disease.[47] In patients with OSA, TNFα is also known to be a very prevalent cytokine, regardless of the patients obesity status, showing it likely is induced by hypoxic events.[24,45] Additionally, other studies have shown that TNFα is likely a key modulator in the inflammation caused by OSA and that it fluctuates based on the severity of the patients OSA. [24,45,46]

IL-1α is considered proinflammatory and shares many of its functions with IL-1ß despite only having a 27% homology to it.[44] That being said one main difference between the two cytokines is that IL-1α is typically viewed as a cytokine released within cells while IL-1ß is released from cells, which if why it often is not viewed as significant in analysis.[40] This being said, it has similar roles as previously discussed in terms of bone resorption and MMP stimulation when produced in excess.[40] In terms of periodontal disease, it is suggested that IL-1α has a mitogenic role in keratinocytes producing the proliferating epithelial effect often seen in periodontitis.[47] In

terms of its role in OSA, IL-1α was seen to be increased in the mucosa of patients with severe, relative to mild, OSA and is thought to be a result of the oxidative stress from the events.[34] Further, it's thought it could be a reason for the fibrosis commonly seen in OSA patients' connective tissue.[34]

IL-1ß is one of the primary pro-inflammatory markers. As suggested previously, IL-1ß has been shown to be released from cells, potentially following apoptosis caused by macrophages, and has been shown to bind to many different cell varieties.[40,47] Like some of the other markers discussed, IL-1ß has been shown to stimulate bone resorption and inhibit bone formation.[40] These actions occur through stimulation of other cells to produce MMPS, plasminogen, prostaglandins, and produce other synergistic pro-inflammatory markers such as TNFα.[40] This being said there is significant evidence in the literature noting the high concentrations of IL-1ß in gingival tissues and GCF in conjunction with attachment loss on teeth.[40,47] Further, these is significant evidence of IL-1ß interactions with other cytokines in the pathogenesis of periodontal disease.[6,33,35,38,40,43,47,48] On study showed that IL-1ß comprised 60% of the osteoclast activating factor and was the most potent factor.[47] Further, this study went on to propose that IL-1ß was the most important cytokine in pathogenesis of periodontal disease.[47] With relation to OSA IL-1ß has been shown to be involved in pathogenesis as well. It is thought that hypoxia in OSA may cause IL-1ß which ultimately will stimulate CD4 T cells.[49] Stimulation of these cells may ultimately lead to production of high levels of ROS and be a leading cause of the inflammation associated with OSA and also potentially decrease defense against bacteria.[49]

IL-6 is also known as one of the primary pro-inflammatory markers. It is produced by many different cells including monocytes, macrophages, fibroblasts, T and B cells, and keratinocytes.[40]

IL-6 is known to have several different actions with one of its main roles being to signal the final maturation of B-cells into plasma cells ultimately causing production of immunoglobulin G (IgG).[40] Further, it is known that IL-6 is also active in bone resorption by potentially autocrine and paracrine function to form and activate osteoclasts.[40] Other sources also note Il-6 ability to promote secretion of MMPs and RANKL.[6] As for its role in periodontal disease, IL-6 is one of the first cytokines to appear and has been said to potentially be the best predictor for periodontal disease.[6,40] Further, IL-6 also been particularly implicated in impairing insulin release and causing a hyper-inflammatory state in patients further increasing their risk for inflammatory diseases.[3] In terms of OSA IL-6 was shown to be correlated with AHI and increased 2 times the normal in patients with OSA versus controls.[41,42,44,49] Although adipose tissue is thought to produce about one third of IL-6, studies have shown that no matter the patients BMI, hypoxia also has a large influence on IL-6 production.[45] This was further explored and confirmed in a study showing that oxidative stress affects nuclear factor kB which works to upregulate IL-6.[34]

Monocyte chemoattract protein 1 (MCP-1) are protein secreted by many cells including macrophages, fibroblasts, epithelial cells, and endothelial cells.[50] While these cells can be quite important for normal biologic functions such as wound healing, and recruitment of inflammatory cells, it can also be involved with several inflammatory disease such as periodontitis.[50] One study showed that there was a correlation with MCP-1 presence and pathogenesis of inflammatory periodontal disease with a very high sensitivity and specificity.[50] Further, another study showed that MCP-1 was higher in GCF in patients with both chronic and aggressive periodontitis and that there was a strong association with the protein following treatment.[48] In terms of sleep apnea, MCP-1 is readily found in atherosclerotic plaques and the vascular intimae space causing

attraction of monocytes to the area.[51] In this situation MCP-1 binds to a protein called CCR2 which assist in chemoattractant functions, and both proteins are found to be increased in more severe apneic patients.[51]

Cortisol has been identified as a potential marker for stress in humans for some times through its release from the hypothalamic-pituitary-adrenal (HPA) axis.[23] Cortisol can change rapidly and be affected by many different factors related to stress and anxiety, among others.[23] Moreover, cortisol has many distinct functions on the body, particularly related to the immune system, and long term production is known to cause deleterious effects on the body.[20] Cortisol has long been implicated in OSA as it has a strong diurnal rhythm and there is thought that disruption of the HPA axis can affect sleep patterns.[20,23] In addition, frequent awakening may cause burst releases of cortisol, and if OSA is left untreated it may result in significantly higher overall cortisol levels.[20] While no referenced studies have been able to correlate cortisol levels with OSA severity, many still believe cortisol levels are related to hypoxia frequency and duration.[20] Interest in cortisol and its effect on periodontal health is currently being explored by several groups as many believe excess production, and the affect it has on one's immune system, likely will make individuals more susceptible to periodontal disease.[28]

Transcriptional activator hypoxia inducible factor 1 alpha (HIF-1α) helps to control genes which encode erythropoietin, vascular endothelial growth factor, and assist with glucose metabolism.[52] In normal conditions, HIF-1α is degraded due to destabilization of prolyl hydroxylases, but under hypoxic conditions the protein is stabilized.[27] This specific protein is therefore directly impacted by hypoxia and oxygen levels in the local area as well as production of ROS species.[27] When this occurs the HIF-1α induces activation that leads to expression of IL-

1ß and MMP-1 which can lead to degradation, as previously discussed.[27] From a periodontal standpoint this protein has attracted attention as it is known that periodontal inflammation can cause localized hypoxia and studies showed that periodontal tissues revealed large amounts of HIF-1α in inflamed tissues compared to controls.[27] Relative to patient with OSA, HIF-1α may have important roles in chronic changes occurring from induced hypoxia following ROS production which alter the local oxygen tension.[15]

Thiobarbituric acid reactive substances (TBARS) is a product of polyunsaturated fatty acid peroxidation which indicates oxidative stress within the patient.[4] TBARS has been popularized because it is seen as one of the first markers that appears upon lipid oxidation in human studies and is a reliable predictor for atheroscelerosis.[25] As we know, oxidative stress in periodontal tissue damage results from host-microbial interactions.[4] Salivary TBARS has been identified as being related to the periodontal status of the patient because of this interaction.[4] On the other hand, TBARS also relates directly to OSA as one study showed a positive correlation between TBARS and AHI, as well as the lowest oxygen saturation.[25]

CHAPTER 2: Cytokine Analysis of Periodontal and OSA patients

Introduction:

In the last decade, there have been much research to understand the potential bidirectional association between periodontitis and obstructive sleep apnea. Efforts to relate these two seemingly different diseases have been challenged by multiple confounding factors, differences in study populations, and biased results. Performing unbiased and valid studies in this field, has proven difficult as it is challenging to determine an associative or causal relationship between major diseases that are influenced so heavily by other systemic and general health conditions.

Periodontal disease is a host-mediated infectious disease of inflammatory nature, usually associated with bacterial biofilm and calculus, characterized by loss of supporting tissues (e.g., alveolar bone and connective tissue).[1] Two examples of a systemic effect of periodontal disease stem from cardiovascular disease (CVD) and diabetes. It has been shown that 91% of individuals with CVD are diagnosed with either moderate or severe periodontal disease.[9] Many investigators are currently working to determine the physiological mechanism, some exploring nitric oxide availability leading to infections by *Porphyromonas gingivalis* or possibly by affecting the ability of LDL to activate macrophages.[11] Further, there has been a long standing association of periodontal disease with diabetes. While it is a well-known fact the prevalence of periodontal disease is higher in those with diabetes, it has been shown to be bidirectional as periodontal disease may increase insulin resistance.[3,12]

While the etiology is much different, obstructive sleep apnea (OSA) has also been shown to affect many different systems through inflammatory pathways. OSA is defined by complete or partial collapse of the upper airway resulting in cessation or reduction of airflow while sleeping.[14] It has been shown that individuals with OSA have higher cardiovascular mortality and morbidity and that there is also increased levels of endothelial damage, increased oxidative stress, and increased sympathetic tone.[22] Additionally, OSA has also been shown to be linked to diabetes by impacting insulin resistance levels.[24]

Technology has evolved in such a way as to allow researchers to take an in-depth look at systemic and local inflammatory makers to assist in understanding the processes underlying these different diseases. These markers can identify biologic mechanisms that may ultimately prove to identify the basis of many other disease conditions. Over the last decade a number of studies have looked specifically at select biomarkers of periodontitis and sleep apnea. A systematic review in 2015 looked at data from six available studies, analyzing IL-1ß, IL-6, IL-21, IL-33 and Pentraxin-3.[53] The results revealed higher salivary levels of IL-6 associated with OSA and also that the odds ratio was 1.65 for patients with chronic periodontal disease to also have sleep apnea.[53] A more recent study found OSA to be associated with higher periodontal indices and IL-1ß.[54] Studies specific to each disease show that many of the same pro-inflammatory and oxidative stress markers are implicated, such as IL-6, but few studies have looked at these markers in individuals with both OSA and periodontal disease. [6,49]

This being said, it seems plausible that these two inflammation-associated diseases influence the course of one another. The aim of this study was to investigate inflammatory aspects of periodontal disease and obstructive sleep apnea in a population of individuals for which the two

vary in severity. In particular, we aimed to investigate how the profile of salivary cytokines associated with periodontal disease is altered by co-existing obstructive sleep apnea (OSA) of different levels of severity.

Materials and Methods:

Study Population and Recruitment:

Participants consisted of a convenience sample population recruited through the Dental Faculty Practice, the Periodontology Clinic, and the Dental Hygiene Program Clinic at University of North Carolina Adams School of Dentistry. Potential participants were informed that the purpose of the study was to understand the association between sleep-disordered breathing (difficulty breathing during sleep) and periodontal disease. Inclusion criteria included a minimum of 10 teeth, 40 to 75 years of age, and being able to speak English. There were no inclusion criteria with regard to gender, ethnicity, race or health status. Written informed consent was obtained prior to data collection. The study was reviewed and approved by the IRB at the University of North Carolina at Chapel Hill.

Demographic, Behavioral and Medical Information:

At the initial visit all patients filled out a series of questionnaires to obtain pertinent demographic, behavioral and medical information. Demographic information included age, gender (M, F), and race (Caucasian and Non-Caucasian). Behavioral information included smoking status (current, former, never), alcohol consumption (number of drinks per day; 0, 1, 2, or >2), bruxism (day/night), and dry mouth frequency (never, monthly, weekly, most days, always) and severity (never, barely detectable, mild, moderate, severe, extreme). Medical information

included BMI, diabetes (none, type 1, type 2), hypertension (yes/no), and a previous diagnosis of OSA (yes/no). Symptoms and risk factors of sleep-disordered breathing were obtained by the STOP-Bang questionnaire (PMID: 18431116), and the Epworth Sleepiness Scale. (PMID: 1519015). Information was recorded by the examiner using TeleForm, an optical scanning system that efficiently transfers information to an electronic format for statistical analysis.

Clinical Periodontal Inflammation:

Probing depths and recession were completed at six sites per tooth for fully erupted teeth using a UNC-15 probe. Level of clinical periodontal inflammation was assessed by bleeding on probing (BOP). A high level of clinical inflammation was defined by a BOP on more than or equal to 20 percent of sites (BOP%≥20); a low level by BOP, on less than 20 percent of sites (BOP%<20)[55]. Based on the clinical evaluation, the ADA/AAP periodontal classification was determined also for each participant.[56] A non-periodontal patient was classified as no bone loss, no CAL, and 2-3mm probing depths. Mild periodontitis was classified as slight bone loss, 1-2mm CAL, and 3-4mm probing depths. Moderate periodontitis was classified as moderate bone loss, 3-4mm CAL, and 5-6mm probing depths. Severe periodontitis was defined by severe bone loss, >5mm CAL, and 7mm or deeper probing.

Severity of Sleep-Disordered Breathing:

Each participant underwent a two-night home sleep apnea test by NovaSom, Inc, (Glen Burnie, Maryland). With this system sensors are placed on the patient's chest, and nose/mouth. With these sensors, the device collects information on blood oxygen saturation, pulse, and

snoring giving estimates of the apnea hypopnea index (AHI), oxygen saturation, and snoring extent. Apnea and hypopnea were scored based on criteria of the American Academy of Sleep Medicine (AASM) for home sleep apnea tests (decrease in peak signal excursion of about 90% for ≥10 seconds and reduction of airflow signal of about 30% or more for ≥10 seconds followed by a ≥3% oxygen desaturation, respectfully). AHI was defined as the number of hypopneas and apneas per hour of recording time. CT90 was defined as the percentage of recording time during which the oxygen saturation was below 90%. For calculation of both the AHI and the CT90, a minimum of 4 hours of recording time was required. To represent the participant's greatest severity of sleep-disordered breathing, the higher of the values from the two nights for both the AHI and of the CT90 was used in the analyses.

Saliva Sampling:

A 3-milliliter unstimulated saliva sample was obtained by spitting. The samples were centrifuged, aliquoted, and coded/stored in a -80 degree Celsius Freezer until analysis.

Cytokine Assays:

SPK Millipore 11-plex saliva magnetic assay was used to identify the levels of IFN-y, IL-4, IL-10, IL-13, TNFα, IL-1α, IL-1ß, IL-6, IL-8, IL-18, and MCP-1. Individual enzyme linked immunosorbent assay (ELISA) kits (Brand R&D Minneapolis, MN) were used to analyze Cortisol, HIF-1α, and TBARS. All processes were completed following the manufactures instructions.

Statistical Analysis:

Descriptive statistics (percentages of participants or means/standard deviations) were calculated for the demographic, behavioral, medical, sleep, and clinical periodontal characteristics of the participants (Tables 1 and 2). For each, descriptive statistics (means/standard errors) also were calculated for participants with high versus low levels of clinical periodontal inflammation (BOP%). Mean differences for the two groups of participants were assessed for statistical significant using unpaired t-tests. Differences in the percentages for the two groups of participants were assessed for statistical significance using Chi-Square tests.

For each biomarker, descriptive statistics of levels present in the saliva were calculated for all participants (means/standard deviations) and for participants with high versus low levels of clinical periodontal inflammation (means/standard errors; Table 3). The unadjusted differences in biomarker levels for the two groups were assessed for statistical significance using unpaired t-tests. Logarithmic transformation (base 10) of biomarker levels was used in all analyses due to the skewed nature of the distribution of non-transformed values.

Subsequent analyses of the biomarker data were conducted to account for potentially confounding factors and to test three hypotheses:

1. Salivary biomarker levels differ for individuals with high (BOP≥20) as compared to low (BOP<20) levels of clinical periodontal inflammation.

2. Salivary biomarker levels increase with the severity of sleep-disordered breathing as assessed by the AHI.

3. The rate of increase (2 above) is greater in individuals with high levels of clinical periodontal inflammation (BOP≥20) than in individual with low levels of periodontal inflammation (BOP<20).

To this end, two multiple-regression models were implemented for each biomarker. The first, full model accounted for level of clinical periodontal inflammation (BOP<20% vs ≥20%), the severity of sleep disordered breathing as assessed by the AHI, the BOP by AHI interaction, and study covariates that previously have been shown to impact biomarker levels (gender, age, race, smoking, alcohol, BMI, presence of diabetes).[44,51,57,58,59,60,61] A second reduced model accounted for the level of clinical periodontal inflammation, the severity of sleep disordered breathing and covariates in the full model that were significant at the $p<0.10$ level. As the BOP by AHI interaction was not significant at the $p<0.10$ level in the full models, it was not included in any of the reduced models. Effects with $p<0.05$ were considered statistically significant. When statistically significant, model adjusted means in the biomarker levels for individuals with high vs low levels of clinical periodontal inflammation were calculated.

Secondary analyses were performed to investigate statistically significant covariates on the biomarker levels. All analyses were performed using SAS 9.4 (SAS Institute, Inc., Cary, NC).

Results:

Data was obtained from a total of 150 participants in a previous study. Of the 150 participants, 134 had saliva collected for analysis, 128 had full periodontal charting with CAL for all sites, and 104 had two nights of sleep data collected. For some participants there was less than 4 hours of recorded data, in which case all data for that night were eliminated from the analyses. Ultimately, after exclusions, 81 participants had complete data including results from

the biomarker assays. 77 of the individuals were included in the analysis for all markers with expection of HIF-1x (n=70), TBARS (n=76), and cortisol (n=76) as interpretable data was received from the assays.

Characteristics of Participants:

Analysis of the demographics of the patients revealed a higher overall percentage of females (58%) than of males (42%) in the study population (Table 1). However, the percentages of females and males did not differ significantly for the two inflammation groups (p=0.81). Additionally, age and race of participants were similar for the entire population (average age 62.9 years old and 81% Caucasian) as for the two inflammation groups (Table 1). The two groups differed significantly on none of the demographic factors (Table 1).

Analysis of the behavioral data revealed similar levels of tobacco smoking (91% never or former smokers) and alcohol use (60.5% yes) for the two inflammation groups as for the entire study population (Table 1). A very low proportion of individuals were current smokers (8.8%). Similar to the demographic factors, no behavioral factor was significantly different for the two groups (Table 1).

Analysis of the collected medical information revealed similar mean levels of BMI (27.5) and measures of the severity of sleep disordered breathing for the two groups of participants (Table 1). A high percentage of participants (88.5%) presented with some level of sleep disordered breathing (AHI>5 events/hr; Table 1). The percentages of participants with AHI values consistent with mild, moderate and severe OSA did not differ for participants with high versus low clinical periodontal inflammation. Similarly, neither the mean AHI (17.4 events/hr) nor mean CT90 (8.4%

of sleep time) differed significantly for the two groups. Interestingly, after eliminating individuals with incomplete data, no participants in the study population were type 1 or type 2 diabetic. As for the demographic and behavioral factors, no medical factors differed significantly for the two groups (Table 1).

Table 1. Demographic, Behavioral and Medical Characteristics of Patients Based on Periodontal Inflammation Status

	All Participants (n=81)	BOP<20% (n=58)	BOP≥20% (n=23)	p-value
	% or Mean/SD	% or Mean/SE	% or Mean/SE	
Demographic				
Gender:				
Female	58.0%	56.9%	60.9%	0.81
Male	42.0%	43.1%	39.1%	
Age	62.9/8.6	62.7/1.1	63.2/1.9	0.82
Race				
Caucasian	81.0%	82.1%	78.3%	0.76
Non-Cauc.	19.0%	17.9%	21.7%	
Behavioral				
Smoking:				
Never	48.8%	47.4%	52.2%	0.92
Former	42.5%	43.9%	39.1%	
Current	8.8%	8.8%	8.7%	
Alcohol:				
Yes	60.5%	56.9%	69.6%	0.33
No	39.5%	43.1%	30.4%	
Medical				
BMI	27.5/6.2	27.9/0.9	27.6/1.0	0.82
Diabetes:				
Type 1				
Type 2				
No	100%	100%	100%	
Sleep-Disordered Breathing:				
AHI	17.4/14.6	16.8/2.2	20.6/3.3	0.28
AHI<5	11.5%	9.5%	12.3%	0.92
5≤AHI<15	47.4%	47.6%	47.4%	
15≤AHI<30	21.8%	19.1%	22.8%	
AHI≥30	19.2%	23.8%	17.5%	
CT90	8.4/16.7	5.2/2.7	10.0/3.9	0.27

Table 1: Participant characteristics for the total study population as well as for the two inflammation groups. BMI, Body Mass Index; AHI, Apnea Hypopnea Index in events/hr; CT90, Cumulative recording Time percentage with SpO2 below 90%

Upon assessment of the participants' periodontal disease, the mean BOP was 18.7% of sites. The periodontal inflammation groups were defined based on BOP% and therefore, the mean BOP% differed significantly for the two groups (p-value 0.04; Table 2). On average, bleeding on probing occurred at twice the percentage of sites in the high (30.8) as compared to the low (13.9)

inflammation group. Further, a very low proportion of participants (6.2%) did not have some periodontal disease (Table 2). Between the two inflammation groups there was a significant difference in the proportions of participants with different severity levels of periodontal disease (p-value 0.01, Table 2). All participants with no or mild periodontitis were in the BOP<20% group and the percentage of participants with severe periodontitis (34.5%) was about half of that of the BOP≥20 group (65.2%).

Table 2. Clinical Periodontal Characteristics of Patients

	All Participants (n=81)	BOP<20% (n=58)	BOP≥20% (n=23)	p-value
BOP	18.7/15.9	13.9/1.6	30.8/3.6	**0.04**
Periodontal Classification (ADA/AAP 1999):				
Non-Perio %	6.2%	8.6%	0%	**0.01**
Mild %	17.3%	24.1%	0%	
Moderate %	33.3%	32.8%	34.8%	
Severe %	43.2%	34.5%	65.2%	

Table 2: Clinical periodontal data shown for the total population and separately for each of the two established inflammatory groups.

Salivary Biomarkers:

Data for each of the fourteen selected biomarkers of inflammation was analyzed to determine whether the concentration levels differed for the two groups of participants defined by bleeding upon probing. Without any adjustment for potentially confounding factors, only IL-6 (p-value 0.01) and IL-10 (p-value 0.02) were found to differ in concentration for the two groups of participants (Table 3).

Table 3. Biomarker levels of Patients Based on Periodontal Inflammation Status

Biomarker	All Patients (n=81)	BOP<20% (n=58)	BOP≥20% (n=23)	Difference	p-value
IL-6	1.258/0.638	1.158/0.103	1.619/0.148	0.461	**0.01***
IL-10	0.656/0.580	0.545/0.094	0.890/0.134	0.345	**0.02***
IL-1ß	1.510/0.704	1.378/0.108	1.709/0.154	0.331	0.05
MCP-1	2.921/0.587	2.825/0.094	2.945/0.134	0.120	0.42
IL-4	1.009/0.258	0.979/0.045	1.094/0.064	0.115	0.10
IL-8	2.803/0.509	2.728/0.082	2.823/0.117	0.095	0.46
HIF-1α	2.30/1.212	2.33/0.188	2.25/0.218	0.08	0.85
IL-13	1.459/0.384	1.466/0.066	1.533/0.094	0.067	0.52
TNF-α	1.186/0.429	1.148/0.072	1.201/0.103	0.053	0.64
IL-1α	3.861/0.446	3.876/0.070	3.846/0.100	0.030	0.78
IFN-ϒ	0.437/0.405	0.442/0.065	0.424/0.092	0.018	0.86
IL-18	2.184/0.598	2.232/0.096	2.244/0.137	0.012	0.94
Cortisol	0.616/0.316	0.653/0.052	0.661/0.074	0.008	0.92
TBARS	0.179/0.055	0.174/0.010	0.176/0.014	0.002	0.91

*Table 3: Results from univariate analyses of biomarkers data. * symbol signifies that difference is statistically significant at the p<0.05 level.*

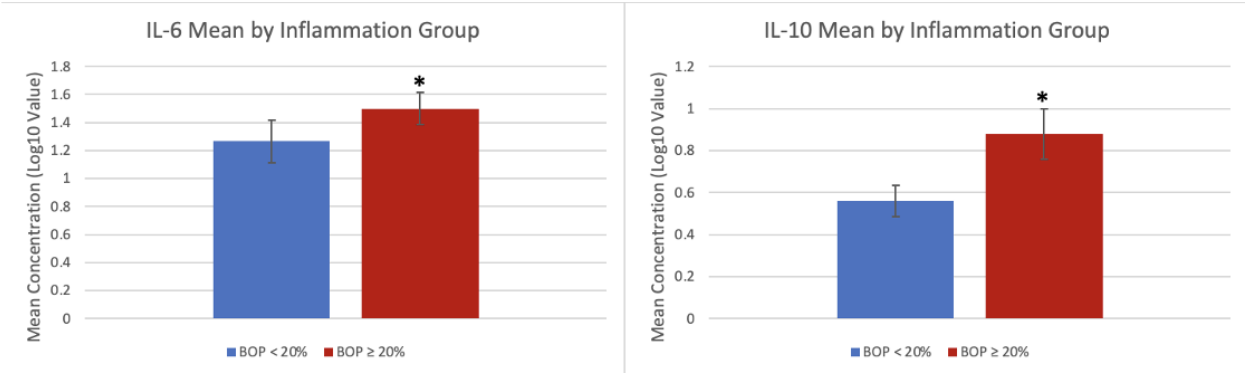


*Figure 1: Adjusted mean levels in Log10 units for IL-6 and IL-10 for participants with high (red) versus low (blue) levels of clinical periodontal inflammation. * symbol signifies that difference is statistically significant at the p< 0.05 level.*

Upon adjustment for all covariates, no differences in the concentration of the biomarkers for the two groups of participants attained statistical significance. (see Table 5, fully adjusted model) However, when irrelevant factors ($p \geq 0.10$) were removed from the analyses,

concentrations of the two biomarkers, IL-6 (p-value 0.02), and IL-10 (p-value 0.02), differed significantly for the two inflammation groups (Table 5, reduced model). Concentration levels (anti-logged values) of IL-6 and IL-10 were on average 137% and 108% higher, respectfully, in participants with high as compared to low clinical periodontal inflammation (Figure 1).

Table 5. Summary of Biomarker Level Results (p-values) from Multi-variable Analyses

	Fully Adjusted Model			Reduced Model	
Biomarker	Inflam. Grp	AHI	Inflam. Grp*AHI	Inflam. Grp	AHI
TNF-α	0.69	0.51	0.56	0.82	0.13
IFN-ϒ	0.17	0.79	0.17	0.43	0.52
IL-1α	0.32	0.61	0.46	0.52	0.40
IL-1ß	0.23	0.93	0.72	0.15	0.14
IL-6	0.08	0.63	0.77	**0.02***	0.08
IL-8	0.85	0.09	0.86	0.76	0.12
IL-18	0.95	0.59	0.80	0.81	0.20
MCP-1	0.85	**0.01***	0.75	0.81	**0.02***
HIF-1α	0.37	0.26	0.38	0.93	0.60
TBARS	0.86	0.63	0.74	0.70	0.41
IL-4	0.71	0.37	0.62	0.25	0.24
IL-13	0.79	0.55	0.55	0.71	0.20
IL-10	0.13	0.76	0.90	**0.02***	0.08
Cortisol	0.49	0.57	0.18	0.52	0.48

*Table 5: P-values for biomarkers based on a fully adjusted model and a reduced model for which confounders were eliminated if they were not significant at the p<0.10 level. Bold with * signifies statistical significance at the p<.05 level.*

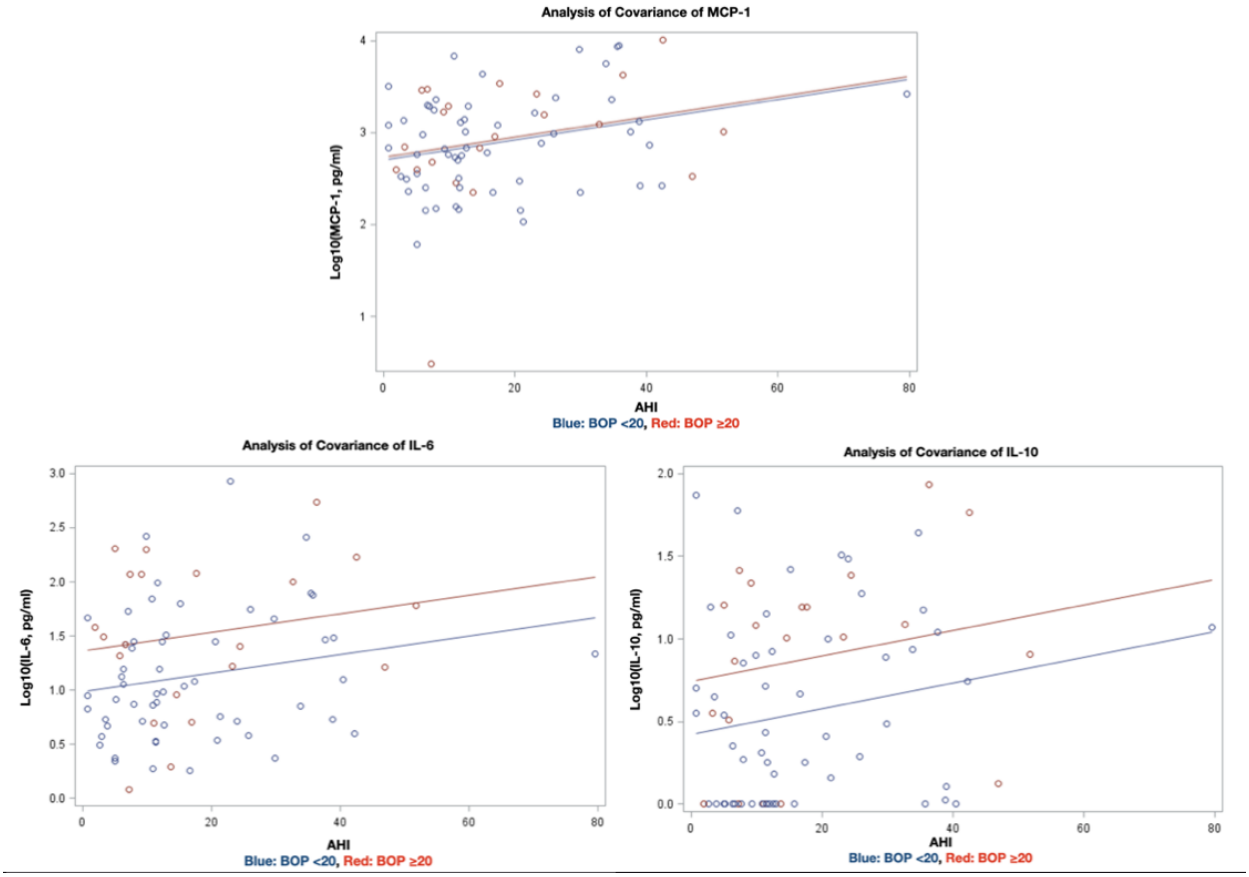


Figure 2: Log10 values of biomarker levels (y-axis) plotted as a function of the severity of sleep-disordered breathing (AHI). Levels of MCP-1 (top) increased with the AHI (p-value 0.01, R^2=0.073). There was a trend for IL-6 levels (lower left) and IL-10 levels (lower right) to increase with the AHI also (p-values 0.08). Data points and regression lines are shown separately for participants with high (red) verus low (blue) levels of clinical periodontal inflammation.

Sleep disordered breathing had little impact on the biomarker levels. For only one biomarker, MCP-1, did concentration levels increase with the frequency of respiratory events during sleep (Table 5). As suggested by Figure 2, the levels of this biomarker (anti-logged values) increased exponentially with the severity of sleep disordered breathing (p-value 0.02). However, the exponent was very low (0.011) and only about 8% of the variability in the logarithmically transformed values could be explained by the AHI. In addition to MCP-1, there was a trend for the levels of IL-6 and IL-10 to increase with AHI but it did not attain statistical significance for either biomarker (p-values 0.08) (Table 5)(Figure 2). For both biomarkers, the exponents were

less than 0.01. As noted above, IL-6 and IL-10 concentration levels were significantly greater for participants with high as compared to low bleeding upon probing. However, there was no evidence to suggest that the exponential growth in concentration differed for participants with high versus low clinical periodontal inflammation. (Table 5).

After adjusting for the effects of BOP and AHI, several factors were identified as having a statistically significant relationships with the concentration levels of the biomarkers. However, in no case did any factor impact the levels of all, or even most, of the biomarkers.

First, gender was shown to have a significant effect on the level of MCP-1 (p-value 0.04), with females having higher levels (Figure 3). On average, the concentration of MCP-1 was 86% higher in females than in males. A gender difference was not observed for any other biomarker.

Secondly, race was shown to affect levels of IL-1α (p-value 0.004), IL-1ß (p-value 0.04), and IL-8 (p-value 0.01) in a consistent manner. 139% higher levels of IL- IL-1α, 177% higher levels of IL-1ß and 143% higher levels of IL-8 were seen in Caucasian, as compared to non-Caucasian, participants. No other biomarkers differed according to the race of the participants.

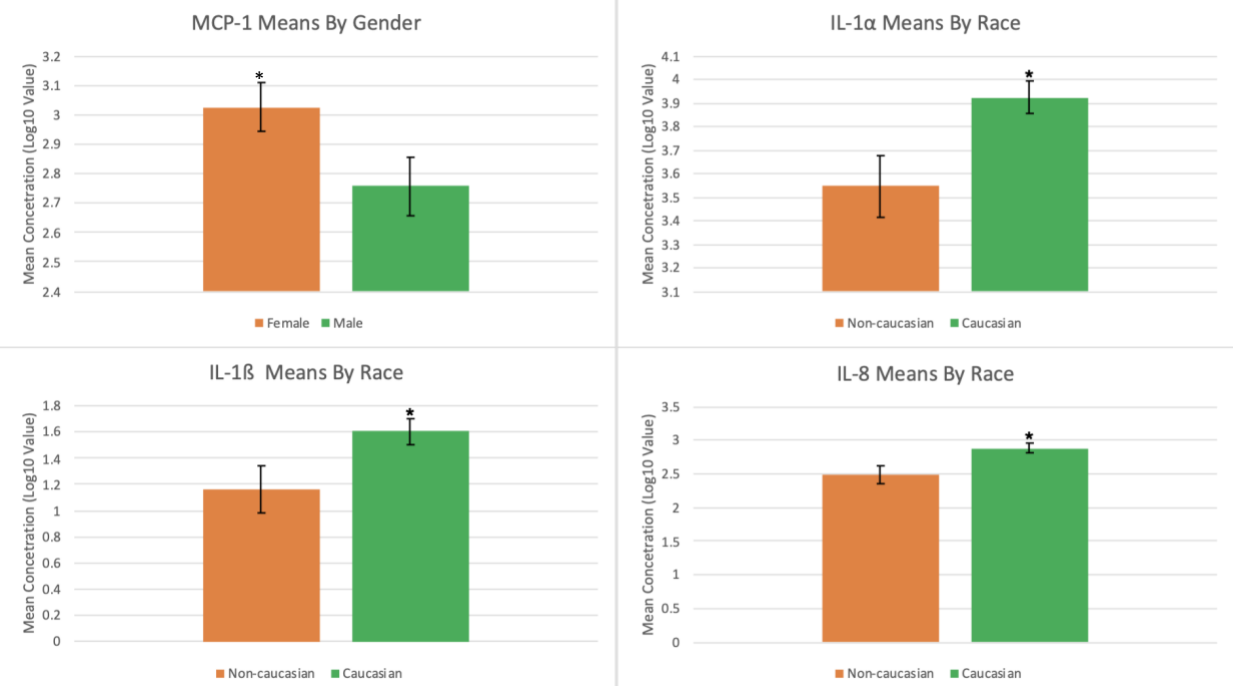


Figure 3: Results of analysis of participants demographic data. In the top left, this shows a signficant relationship (p-value 0.04) of MCP-1 in female participants. In the top right, this shows a signficant relationship (p-value 0.004) of IL-1α concentration in caucasin participants. In the bottom left, this shows a signficant relationship (p-value 0.04) of IL-1ß concentration in caucasin participants. In the bottom right, this shows a signficant relationship (p-value 0.01) of IL-8 concentration in caucasian participants.

Third, it was shown that concentration levels of IL-1ß increased exponentially with participants' BMI (p-value 0.01) (Figure 4). However, the exponent was low (0.023) and only 4% of the variability in the logarithmically transformed values could be explained by the BMI. The participants' BMI did not affect the concentration levels of any other biomarker in the study.

Fourth, age of the participants was shown to have an effect on MCP-1 concentration levels (p-value 0.02). The levels increased exponentially with age (exponent = 0.024), which explained 12% of the variability in the logarithmically transform values. (Figure 4) The participants' age did not affect the concentration levels of any other biomarker in the study.

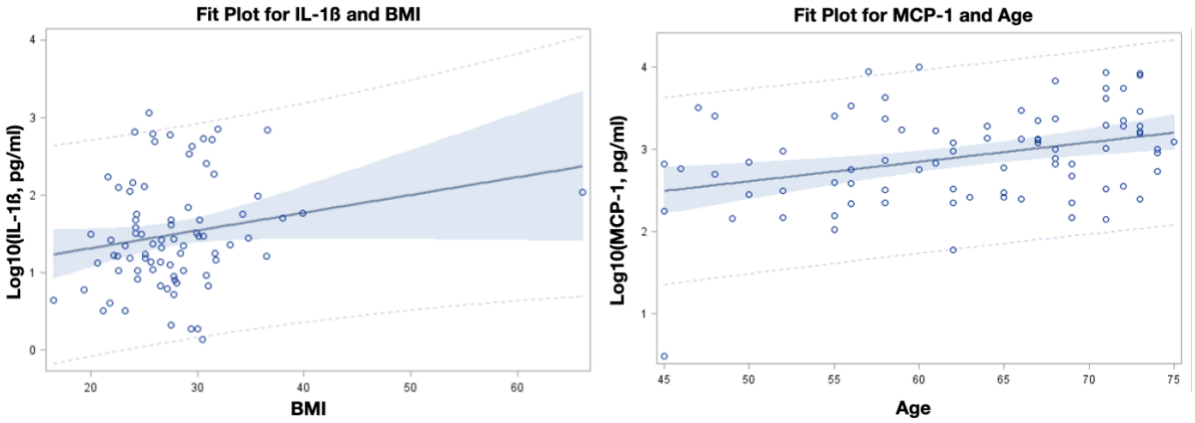


Figure 4: Left panel: Exponential relationship between IL-1ß and body mass index (BMI)(p-value 0.01; R2=0.14). Right panel: Exponential relationship between MCP-1 and age of the participant (p-value 0.02, R2=0.12). Shaded areas of graphs represent 90% confidence intervals.

Although no other potentially confounding factors were found to be statistically significant in our analysis, there was a trend for smoking status to impact the concentration levels of cortisol (p-value 0.12). As compared to never smokers, cortisol levels were progressively higher for former and current smokers, respectively.

Discussion:

From biomarker analyses of saliva of the participants our data revealed several significant findings. Firstly, we found an association of the level of clinically observed inflammation, based on percentage of sites with BOP, with IL-6 and IL-10. Levels of these biomarkers were higher for participants with high as compared to low clinically observed periodontal inflammation. Secondly, we found that the severity of sleep disordered breathing, based on the AHI, affected the levels of cytokine MCP-1, and there was a trend to affect both IL-6, and IL-10 also. In all three cases, levels of the biomarkers increased with the frequency of respiratory events per hour during sleep. Finally, we found that select cytokine levels were

significantly affected by several covariates considered in the analyses: race (IL-1ß, IL-1α, and IL-8, all higher in Caucasian than in non-Caucasian participants), age (MCP-1, increasing with age), and BMI (IL-1ß, increasing with BMI). In addition, higher levels of MCP-1 were observed in female than in male participants.

Salivary biomarker levels differ for individuals with high (BOP≥20) as compared to low (BOP<20) levels of clinical periodontal inflammation.

This hypothesis was supported by data from two of the 14 biomarkers selected for study, IL-6 and IL-10. Consistent with the literature, there has been years of research on the involvement of IL-6 and IL-10 in periodontal disease.[40,36] While IL-6 typically is considered pro-inflammatory and IL-10 anti-inflammatory, these cytokines are products of TH2 cells, and therefore can increase in parallel.[6] Additionally, studies have shown that levels of these two cytokines are positively correlated in obese subjects, and that this could also potentially relate to their gingival index regardless of their BMI.[62] Dogan et al. proposed that these cytokines often have co-varying levels, especially in obese individuals, as TH2 cells will produce IL-10 to moderate the effects of IL-6 in the presence of high levels of inflammation.[62] The data of the present study is consistent with this hypothesis. Overall our population was overweight (mean BMI=27.5) and exhibited varying degrees of both localized and systemic inflammation.

Salivary biomarker levels increase with the severity of sleep-disordered breathing as assessed by the AHI.

This hypothesis was supported by data from one of the 14 biomarkers selected for analysis, MCP-1. In other studies, MCP-1 has been found to be increased in patients with OSA in proportion to the severity of OSA.[51,63] MCP-1 is a primary recruiter of monocytes to areas of injury and inflammation, specifically of endothelial cells.[51] A relationship to OSA potentially stems from monocytes activated by damaged endothelial cells in response to the chronic intermittent hypoxia of sleep disordered breathing.[51] Our study confirms these previous findings of the effect of AHI on expression of MCP-1. In addition to MCP-1, there was a trend for both cytokines IL-6 and IL-10 to increase with the AHI. While studies have shown an association between AHI and IL-6 ([64]), the opposite effect on IL-10 has been reported typically.[24] Because most participants had some level of sleep disordered breathing, it is possible that its additional contribution to inflammation resulted in a progressive elevation, rather than a depression, in IL-10 levels as has been described before.[62] Based on this possibility, one would predict that the rate at which IL-10 levels increase with elevations in the AHI would be greater for participants with high as compared to low clinical periodontal inflammation. Such a difference, however, was not observed.

The rate of increase with the AHI is greater in individuals with high levels of clinical periodontal inflammation (BOP≥20) than in individual with low levels of periodontal inflammation (BOP<20).

This hypothesis was not supported by data from any of the 14 biomarkers selected for analysis. In no case did the biomarker levels appear to be greater than the sum of the effects attributable to clinical periodontal inflammation and to the AHI alone. While we hypothesized the presence of an interactive effect of the two sources of inflammation, it was not observed. To

the author's knowledge, there are only three publications that address biomarker levels in individuals with varying degrees of periodontal disease and OSA. None of these appear to look closely, or report, an interactive effects of the severity of the periodontal disease and that of OSA on biomarker levels.[54,65,66] This suggests that while both diseases contribute to an overall systemic effect detectable in saliva, they do not influence the production of the biomarkers interactively. The possibility of an interaction is an interesting question to explore in future research with larger groups of participants.

Other factors affecting biomarker levels

Our study revealed the impact of only a few demographic and behavioral characteristics on select biomarkers levels. Firstly, there was a significant increase in MCP-1 in female individuals in our study. To the authors knowledge no other study has evaluated gender differences on MCP-1 levels in individuals with varying degrees of periodontal inflammation and/or of OSA. However, evidence suggests that gender may impact the level of biomarker production.[57] While there is some controversy on the subject, some researchers believe that the different sex hormones impact cytokine production by blood monocytes.[57] Additionally, one study suggested that female sex hormones may decrease MCP-1, thereby providing a cardioprotective effect that is not observed in postmenopausal women.[67] Interestingly, this effect potentially may provide some explanation for the results seen in our study based on the average age (62.9) and provided demographic information of the participants (58% women).

Secondly, several cytokines were shown to differ in concentration according to race. While the effect has not been extensively explored, there is anecdotal data to suggest that race

can impact biomarker concentrations. One example comes from a study in which researchers identified the response of IFNα, IL-2, and IFN-ϒ to vaccines, which varied according to self-reported race.[58] In the present study, Caucasian participants showed significantly higher levels of IL-1ß, IL-1α, and IL-8 than did African Americans or Hispanic participants. While the authors were not able to find similar reports in the literature, this again may be an interesting area for future studies.[58]

Third, another significant effect pertains to age. In particular, levels of MCP-1 increased with the participants' age. It has been shown that aging promotes an increase in inflammatory markers due to the preservation of memory T cell populations.[68] In this regard, MCP-1 attracts memory T-cells to sites of inflammation, and has been shown previously to be increased in aging subject populations.[69] On the other hand, studies of individuals with severe apnea have not found an association between age and MCP-1.[51] More research is needed in this area to determine the effect of aging on inflammatory marker production.

Fourth, another finding of our study relates to the impact of participants' BMI on levels of IL-1ß. The effect of obesity and increased BMI on the production of inflammatory markers is well established.[44,62,64] Most research has focused on the production of IL-6 and TNF-α in relation to the effect of BMI.[70] In addition, these cytokines play a role in increased insulin resistance which, in turn, may contribute to increased BMI.[71] This being said, often times IL-1ß is produced in concert with IL-6 and TNF-α.[6] Further, other studies have confirmed increased levels of IL-1ß in obese individuals.[72]

Finally, while not statistically significant there was a trend for a dose dependent effect of smoking on cortisol. While studying cortisol can prove challenging due to rapidly changing levels

in response to physiological and physical stressors, this effect of smoking has been shown elsewhere in the literature.[73,74] While smoking is known to affect the hypothalamic-pituitary-adrenal axis, the specific pathway for an effect of smoking is still up for debate in the literature, but others have seen similar trends as our study.[74] This relationship between cortisol with smoking is becoming well accepted and may be an area of future interest.

Limitations:

This study had several limitations. Firstly, due to the nature of participant recruitment there was potential selection bias for individuals who previously had been treated for periodontal disease and who were being seen periodically in a periodontal or hygiene clinic. In theory, this could have affected the cytokine concentrations as the patients' recorded periodontal status may have not accurately reflected their inflammatory status at the time of saliva collection. While clinical attachment loss provides a significant source of data for classification it is indicative of chronic disease more so than the level of inflammation. In theory, having all data collected at a patient's initial periodontal exam before any treatment would be the most ideal, but may not be feasible for a sizable study. Further, using other classifications for bleeding on probing could have better defined our study groups for degree of inflammation.

Due to the nature of recruitment it was likely that many patients suspected or knew, based on a previous sleep study, that they had OSA. It is possible that the opportunity to have a home sleep test attracted some participants to the study. Having individuals who were being treated with CPAP may have affected the concentrations of biomarkers, particularly for more severe cases of sleep apnea.[75] Many sources report that first night home sleep data may be

inaccurate due to patient stress, comfort, and familiarity using the machine.[76] In this case, the AHI may be underestimated on the first night of testing. It is also possible that the patient slept better on the first night, or less than 4 hours of data were recorded on the second night. For this reason, we used the higher of AHI values of the two nights to represent the severity of sleep disordered breathing in the analyses.

There are also errors that could occur during saliva sampling. While saliva use is a viable technique for measuring cytokines, inflammatory markers, and oxidative stress, there is some debate as to the specificity of the data collected in comparison to that from the gingival crevicular fluid (GCF) or the patient's blood.[77] While saliva samples are larger in comparison to GCF, they are also more diluted. While this is equated for, there is still the possibility of pulling an inaccurate sample, and there is much debate in the literature as to whether saliva is inferior to GCF.[77] In addition, there could be issues with collection of the samples, processing the samples in the lab, storage of the samples, and with many other procedural aspects. Further, many times samples are run twice in order to confirm the accuracy of the lab work and eliminate potential miscues in the data.

The results of our study should be interpreted as exploratory. No other studies to the authors' knowledge have assessed the high number of biomarkers in participants with varying degrees of periodontal inflammation and sleep disordered breathing. Based on the number of biomarkers, if Bonferroni correction had been used for multiple testing of the data, a p-value of 0.0036 would have been needed to show statistical significance. Additional studies are needed to confirm the findings of this paper.

CHAPTER 3: FUTURE DIRECTIONS

This exploratory investigation opens up the potential for larger clinical studies to clarify the association between periodontal disease and sleep apnea reported in the literature. While this study provides insight into an exploratory and hypothetical nature, larger studies with a more less diverse population may better be able to confirm some of the findings of this paper. Further, a study with careful documentation of more inflammatory traits may be needed in order to better categorize patients conditions at the time of the study, such as multiple BOP recordings. Future studies may also look into more factors directly related to MCP-1 and the cascade of inflammatory markers that it influences as a potential area of development, along with several other inflammatory markers which may be of importance.

APPENDIX

Table 4a. Multi-variable analyses for TNF-α (n=77)

	Fully Adjusted Model					Reduced Model				
Source	df	Type III SS	Mn Sq	FValue	Pr>F	df	Type III SS	Mn Sq	FValue	Pr>F
Grp	1	0.030	0.030	0.16	0.69	1	0.010	0.010	0.05	0.82
AHI3	1	0.082	0.082	0.44	0.51	1	0.416	0.416	2.33	0.13
Grp*AHI3	1	0.066	0.066	0.35	0.56					
gender	1	0.006	0.006	0.03	0.87					
age	1	0.210	0.210	1.11	0.30					
race	1	0.129	0.129	0.68	0.41					
smoking	2	0.175	0.175	0.46	0.63					
alcohol	1	0.278	0.278	1.48	0.23					
BMI	1	0.069	0.069	0.36	0.55					

Table 4b. Multi-variable Analyses for IFN-ϒ (n=77)

	Fully Adjusted Model					Reduced Model				
Source	df	Type III SS	Mn Sq	FValue	Pr>F	df	Type III SS	Mn Sq	FValue	Pr>F
Grp	1	0.262	0.262	1.97	0.17	1	0.081	0.081	0.63	0.43
AHI3	1	0.009	0.009	0.07	0.79	1	0.053	0.053	0.42	0.52
Grp*AHI3	1	0.258	0.257	1.94	0.17					
gender	1	0.255	0.255	1.92	0.17					
age	1	0.016	0.016	0.12	0.73					
race	1	0.013	0.013	0.10	0.75					
smoking	2	0.389	0.389	1.46	0.24					
alcohol	1	0.154	0.154	1.16	0.29					
BMI	1	0.000	0.000	0.00	0.99					

Table 4c. Multi-variable Analyses for IL-1α (n=77)

	Fully Adjusted Model					Reduced Model				
Source	df	Type III SS	Mn Sq	FValue	Pr>F	df	Type III SS	Mn Sq	FValue	Pr>F
Grp	1	0.181	0.181	1.00	0.32	1	0.084	0.084	0.42	0.52
AHI3	1	0.049	0.049	0.27	0.61	1	0.141	0.141	0.71	0.40
Grp*AHI3	1	0.103	0.103	0.57	0.46					
gender	1	0.185	0.185	1.02	0.32					
age	1	0.146	0.146	0.81	0.37					
race	1	0.734	0.734	4.05	**0.05**	1	1.59426	1.59426	9.09	**0.004**
smoking	2	0.211	0.211	0.58	0.56					
alcohol	1	0.001	0.001	0.05	0.82					
BMI	1	0.158	0.158	0.87	0.36					

Table 4d. Multi-variable Analyses for IL-1ß (n=77)

	Fully Adjusted Model					Reduced Model				
Source	df	Type III SS	Mn Sq	FValue	Pr>F	df	Type III SS	Mn Sq	FValue	Pr>F
Grp	1	0.063	0.063	1.45	0.23	1	0.991	0.991	2.15	0.15
AHI3	1	0.003	0.003	0.01	0.93	1	1.043	1.043	2.26	0.14
Grp*AHI3	1	0.056	0.056	0.13	0.72					
gender	1	0.215	0.215	0.50	0.48					
age	1	0.060	0.060	0.14	0.71					
race	1	1.374	1.374	3.16	0.08	1	1.89708	1.89708	4.5	**0.04**
smoking	2	1.066	1.066	1.23	0.30					
alcohol	1	0.575	0.575	1.32	0.25					
BMI	1	3.501	3.501	8.06	**0.01**	1	3.151059	3.151059	7.48	**0.01**

Table 4e. Multi-variable Analyses for IL-4 (n=77)

	Fully Adjusted Model					Reduced Model				
Source	df	Type III SS	Mn Sq	FValue	Pr>F	df	Type III SS	Mn Sq	FValue	Pr>F
Grp	1	0.011	0.011	0.14	0.71	1	0.090	0.090	1.34	0.25
AHI3	1	0.062	0.062	0.81	0.37	1	0.094	0.094	1.40	0.24
Grp*AHI3	1	0.019	0.019	0.25	0.62					
gender	1	0.024	0.024	0.32	0.57					
age	1	0.000	0.000	0.00	0.98					
race	1	0.004	0.004	0.05	0.83					
smoking	2	0.006	0.006	0.04	0.96					
alcohol	1	0.003	0.003	0.04	0.85					
BMI	1	0.015	0.015	0.19	0.66					

Table 4f. Multi-variable Analyses for IL-6 (n=77)

	Fully Adjusted Model					Reduced Model				
Source	df	Type III SS	Mn Sq	FValue	Pr>F	df	Type III SS	Mn Sq	FValue	Pr>F
Grp	1	1.305	1.305	3.20	0.08	1	2.205	2.205	5.89	**0.02**
AHI3	1	0.096	0.096	0,23	0.63	1	1.192	1.192	3.18	0.08
Grp*AHI3	1	0.035	0.035	0.09	0.77					
gender	1	0.014	0.014	0.03	0.85					
age	1	0.611	0.611	1.50	0.23					
race	1	0.066	0.066	0.16	0.69					
smoking	2	0.274	0.274	0.34	0.72					
alcohol	1	0.660	0.660	1.62	0.21					
BMI	1	0.083	0.083	0.20	0.65					

Table 4g. Multi-variable Analyses for IL-8 (n=77)

	Fully Adjusted Model					Reduced Model				
Source	df	Type III SS	Mn Sq	FValue	Pr>F	df	Type III SS	Mn Sq	FValue	Pr>F
Grp	1	0.096	0.096	0.38	0.54	1	0.025	0.025	0.10	0.76
AHI3	1	0.138	0.138	0.55	0.46	1	0.663	0.663	2.53	0.12
Grp*AHI3	1	0.082	0.082	0.33	0.57					
gender	1	0.473	0.473	0.51	0.17					
age	1	0.126	0.126	4.37	0.48					
race	1	1.093	1.093	1.89	**0.04**	1	1.655452	1.655452	6.91	**0.01**
smoking	2	0.092	0.092	0.18	0.83					
alcohol	1	0.010	0.010	0.04	0.84					
BMI	1	0.262	0.262	1.05	0.31					

Table 4h. Multi-variable Analyses for IL-10 (n=77)

	Fully Adjusted Model					Reduced Model				
Source	df	Type III SS	Mn Sq	FValue	Pr>F	df	Type III SS	Mn Sq	FValue	Pr>F
Grp	1	0.755	0.755	2.37	0.13	1	1.530	1.530	4.99	**0.03**
AHI3	1	0.030	0.030	0.10	0.76	1	0.979	0.979	3.19	0.08
Grp*AHI3	1	0.005	0.005	0.01	0.90					
gender	1	0.329	0.329	1.03	0.31					
age	1	0.006	0.006	0.02	0.90					
race	1	0.438	0.438	1.37	0.25					
smoking	2	0.596	0.596	0.93	0.40					
alcohol	1	1.156	1.156	3.62	0.06					
BMI	1	0.427	0.427	1.34	0.25					

Table 4i. Multi-variable Analyses for IL-13 (n=77)

	Fully Adjusted Model					Reduced Model				
Source	df	Type III SS	Mn Sq	FValue	Pr>F	df	Type III SS	Mn Sq	FValue	Pr>F
Grp	1	0.011	0.011	0.07	0.79	1	0.020	0.020	0.14	0.71
AHI3	1	0.057	0.057	0.36	0.55	1	0.249	0.249	1.70	0.20
Grp*AHI3	1	0.057	0.057	0.36	0.55					
gender	1	0.100	0.100	0.64	0.43					
age	1	0.014	0.014	0.09	0.77					
race	1	0.063	0.063	0.40	0.53					
smoking	2	0.180	0.180	0.57	0.57					
alcohol	1	0.226	0.226	1.44	0.23					
BMI	1	0.068	0.068	0.43	0.51					

Table 4j. Multi-variable Analyses for IL-18 (n=77)

	Fully Adjusted Model					Reduced Model				
Source	df	Type III SS	Mn Sq	FValue	Pr>F	df	Type III SS	Mn Sq	FValue	Pr>F
Grp	1	0.001	0.001	0.00	0.95	1	0.020	0.020	0.06	0.81
AHI3	1	0.099	0.099	0.29	0.59	1	0.587	0.587	1.64	0.20
Grp*AHI3	1	0.021	0.021	0.07	0.80					
gender	1	1.200	1.200	3.58	0.06					
age	1	0.112	0.112	0.33	0.57					
race	1	0.928	0.928	2.77	0.10					
smoking	2	1.223	1.223	1.82	0.17					
alcohol	1	0.035	0.035	0.10	0.75					
BMI	1	0.892	0.892	2.66	0.11					

Table 4k. Multi-variable Analyses for MCP-1 (n=77)

	Fully Adjusted Model					Reduced Model				
Source	df	Type III SS	Mn Sq	FValue	Pr>F	df	Type III SS	Mn Sq	FValue	Pr>F
Grp	1	0.011	0.011	0.04	0.85	1	0.037	0.037	0.06	0.81
AHI3	1	0.811	0.811	2.79	**0.01**	1	1.966	1.966	6.28	**0.01**
Grp*AHI3	1	0.031	0.031	0.11	0.75					
gender	1	1.181	1.181	4.07	**0.05**	1	0.866	0.866	4.45	**0.04**
age	1	1.092	1.092	3.76	**0.06**	1	1.621	1.621	5.78	**0.02**
race	1	0.020	0.020	0.07	0.80					
smoking	2	0.150	0.150	0.26	0.77					
alcohol	1	0.051	0.051	0.18	0.68					
BMI	1	0.071	0.071	0.25	0.62					

Table 4l. Multi-variable Analyses for HIF-1α (n=70)

	Fully Adjusted Model					Reduced Model				
Source	df	Type III SS	Mn Sq	FValue	Pr>F	df	Type III SS	Mn Sq	FValue	Pr>F
Grp	1	1.121	1.121	0.82	0.37	1	0.011	0.011	0.01	0.93
AHI3	1	1.818	1.818	1.32	0.26	1	0.433	0.433	0.28	0.60
Grp*AHI3	1	1.096	1.096	0.8	0.38					
gender	1	2.601	2.601	1.89	0.17					
age	1	0.010	0.010	0.01	0.93					
race	1	0.945	0.945	0.69	0.41					
smoking	2	7.338	7.338	2.67	0.08	1	7.889	3.944	2.7	0.08
alcohol	1	2.826	2.826	2.06	0.16					
BMI	1	1.565	1.565	1.14	0.29					

Table 4m. Multi-variable Analyses for TBARS (n=76)

	Fully Adjusted Model					Reduced Model				
Source	df	Type III SS	Mn Sq	FValue	Pr>F	df	Type III SS	Mn Sq	FValue	Pr>F
Grp	1	0.00008	0.00008	0.03	0.86	1	0.0004	0.0004	0.15	0.70
AHI3	1	0.0007	0.0007	0.23	0.63	1	0.002	0.002	0.69	0.41
Grp*AHI3	1	0.0003	0.0003	0.11	0.74					
gender	1	0.002	0.0020	0.67	0.42					
age	1	0.001	0.001	0.37	0.54					
race	1	0.0006	0.0006	0.22	0.64					
smoking	2	0.0008	0.0008	0.14	0.87					
alcohol	1	0.0005	0.0005	0.18	0.67					
BMI	1	0.0006	0.0006	0.22	0.64					

Table 4n. Multi-variable Analyses for Cortisol (n=76)

	Fully Adjusted Model					Reduced Model				
Source	df	Type III SS	Mn Sq	FValue	Pr>F	df	Type III SS	Mn Sq	FValue	Pr>F
Grp	1	0.045	0.045	0.49	0.49	1	0.041	0.041	0.41	0.52
AHI3	1	0.031	0.031	0.33	0.57	1	0.048	0.048	0.49	0.48
Grp*AHI3	1	0.172	0.172	1.84	0.18					
gender	1	0.029	0.029	0.31	0.58					
age	1	0.065	0.065	0.70	0.41					
race	1	0.111	0.111	1.19	0.28					
smoking	2	0.681	0.681	3.65	**0.03**	2	0.419	0.210	2.17	0.12
alcohol	1	0.192	0.192	2.06	0.16					
BMI	1	0.052	0.052	0.55	0.46					

Figure 2.1:

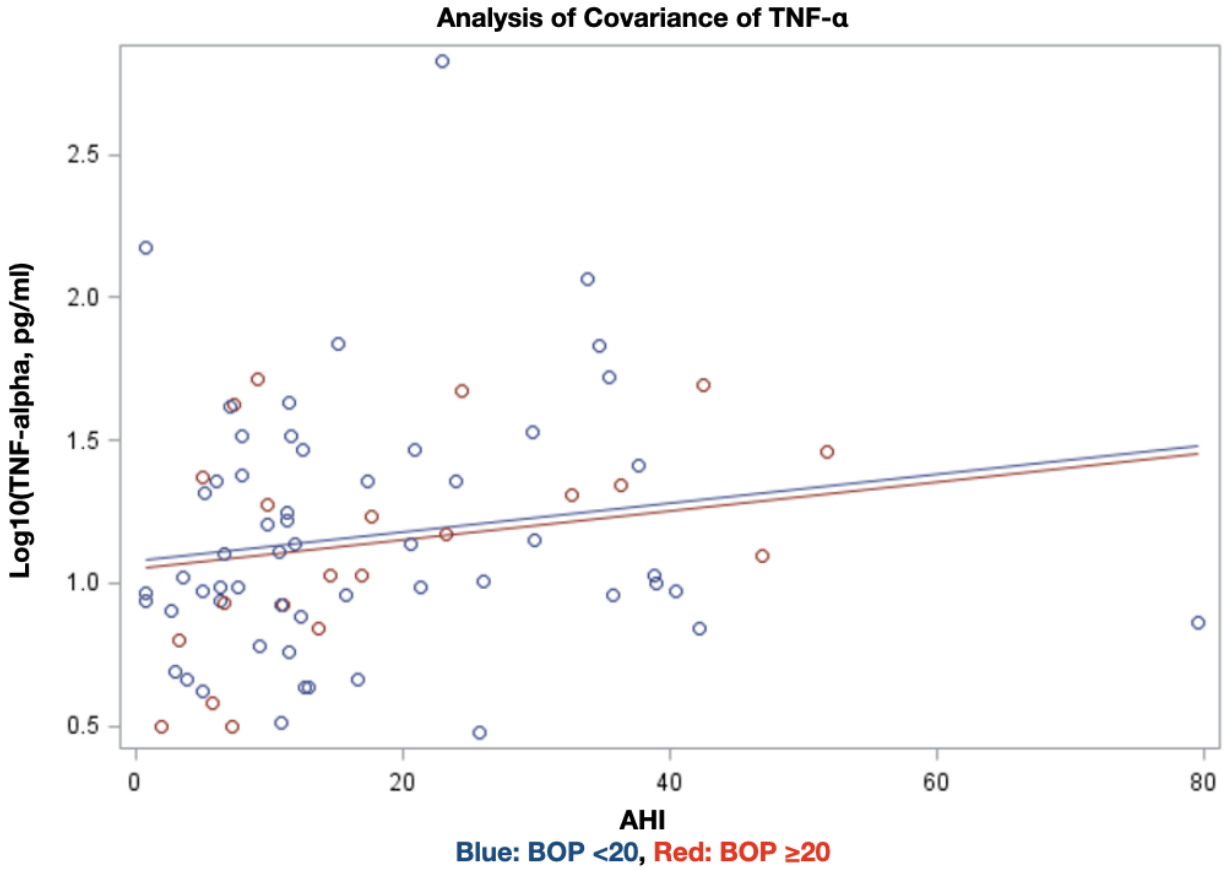

Analysis of Covariance of TNF-α
Log10(TNF-alpha, pg/ml)
2.5
2.0
1.5
1.0
0.5
0
20
40
60
80
AHI
Blue: BOP <20, Red: BOP ≥20

Figure 2.2:

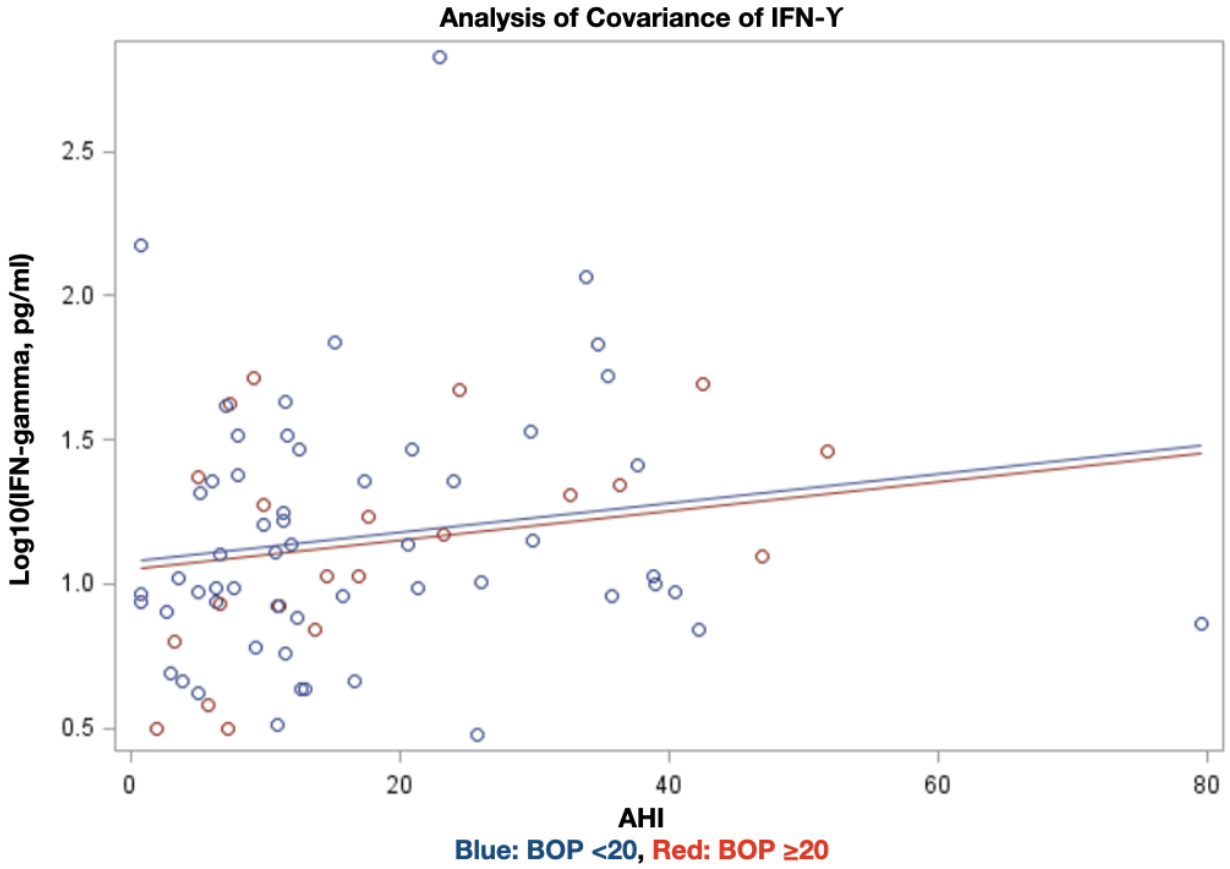

Analysis of Covariance of IFN-Y
Log10(IFN-gamma, pg/ml)
2.5
2.0
1.5
1.0
0.5
0
20
40
60
80
AHI
Blue: BOP <20, Red: BOP ≥20

Figure 2.3:

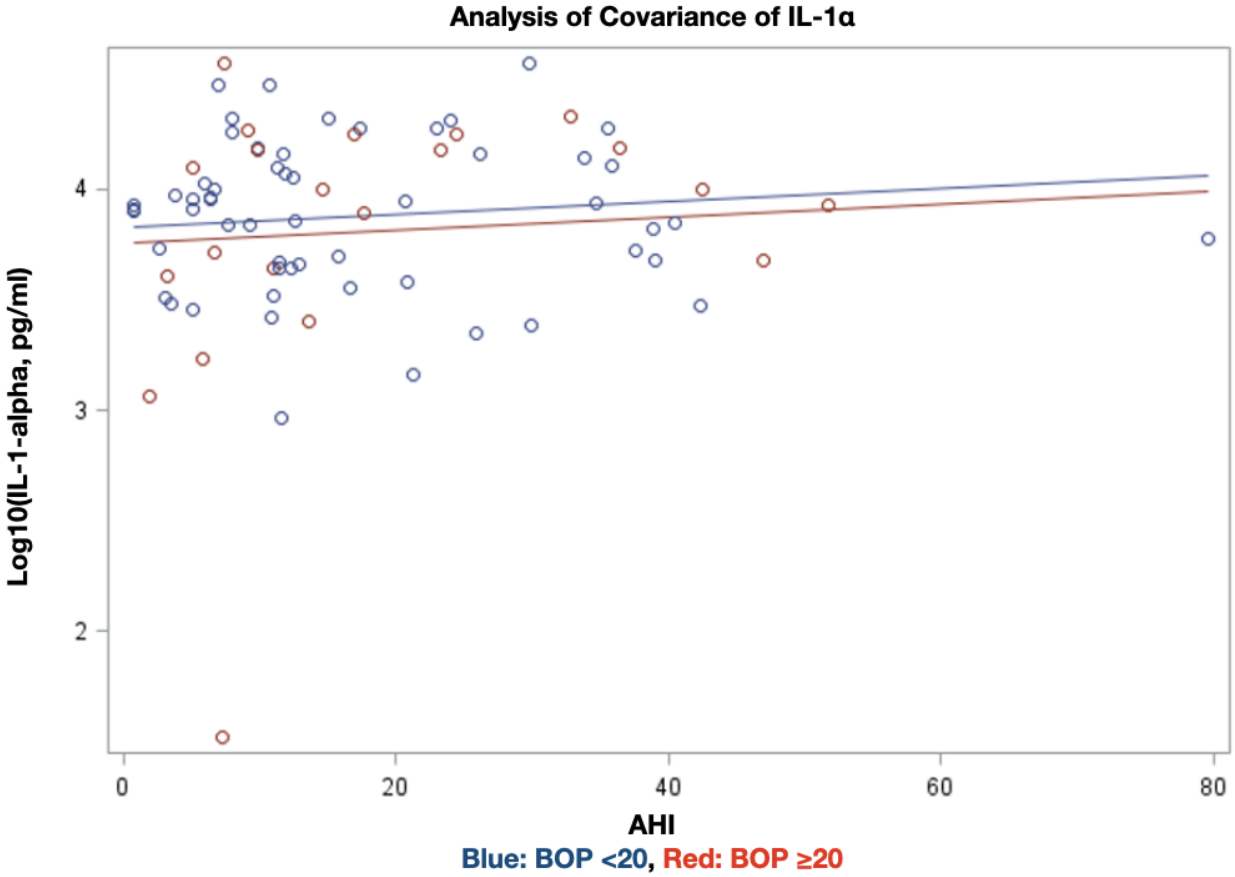

Analysis of Covariance of IL-1α
Log10(IL-1-alpha, pg/ml)
4
3
2
0
20
40
60
80
AHI
Blue: BOP <20, Red: BOP ≥20

Figure 2.4:

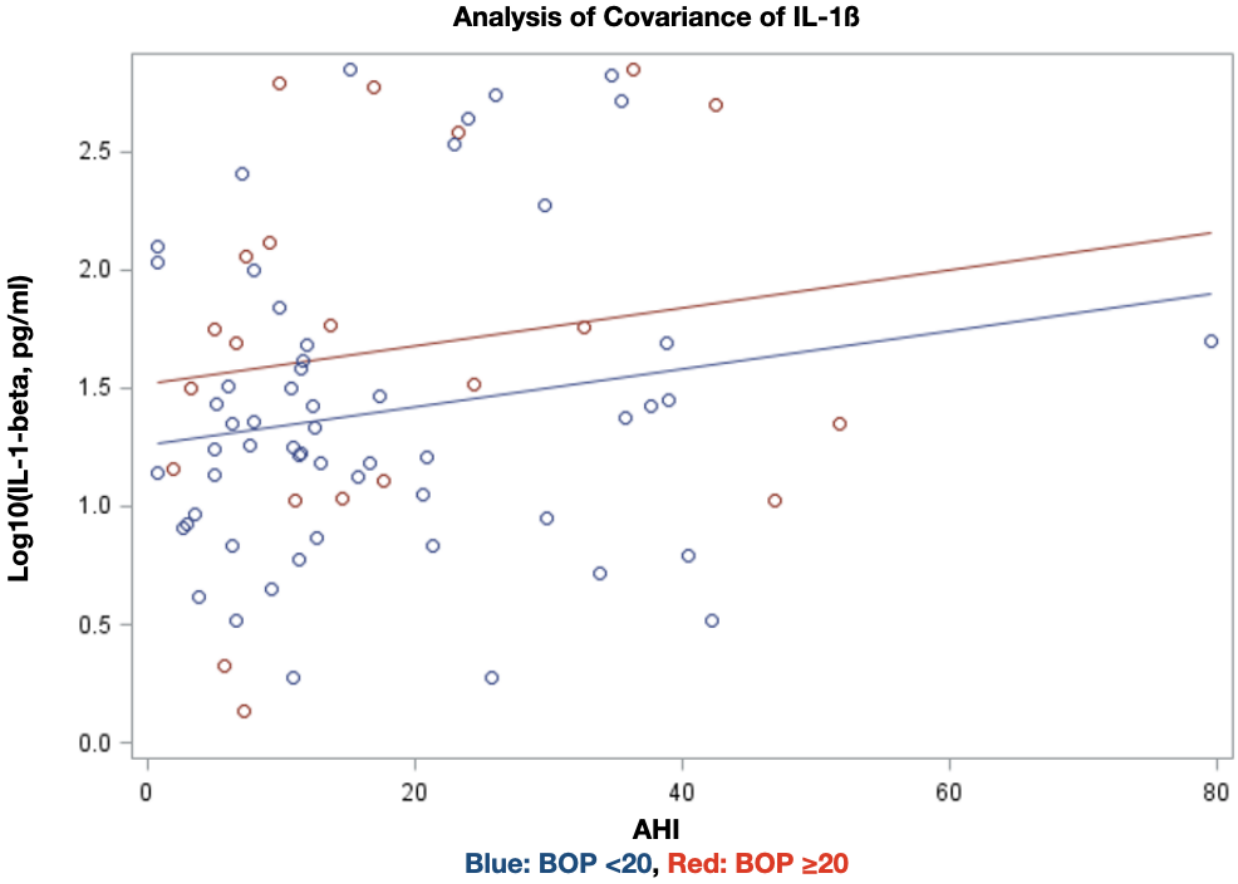

Analysis of Covariance of IL-1ß
Log10(IL-1-beta, pg/ml)
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
AHI
Blue: BOP <20, Red: BOP ≥20

Figure 2.5:

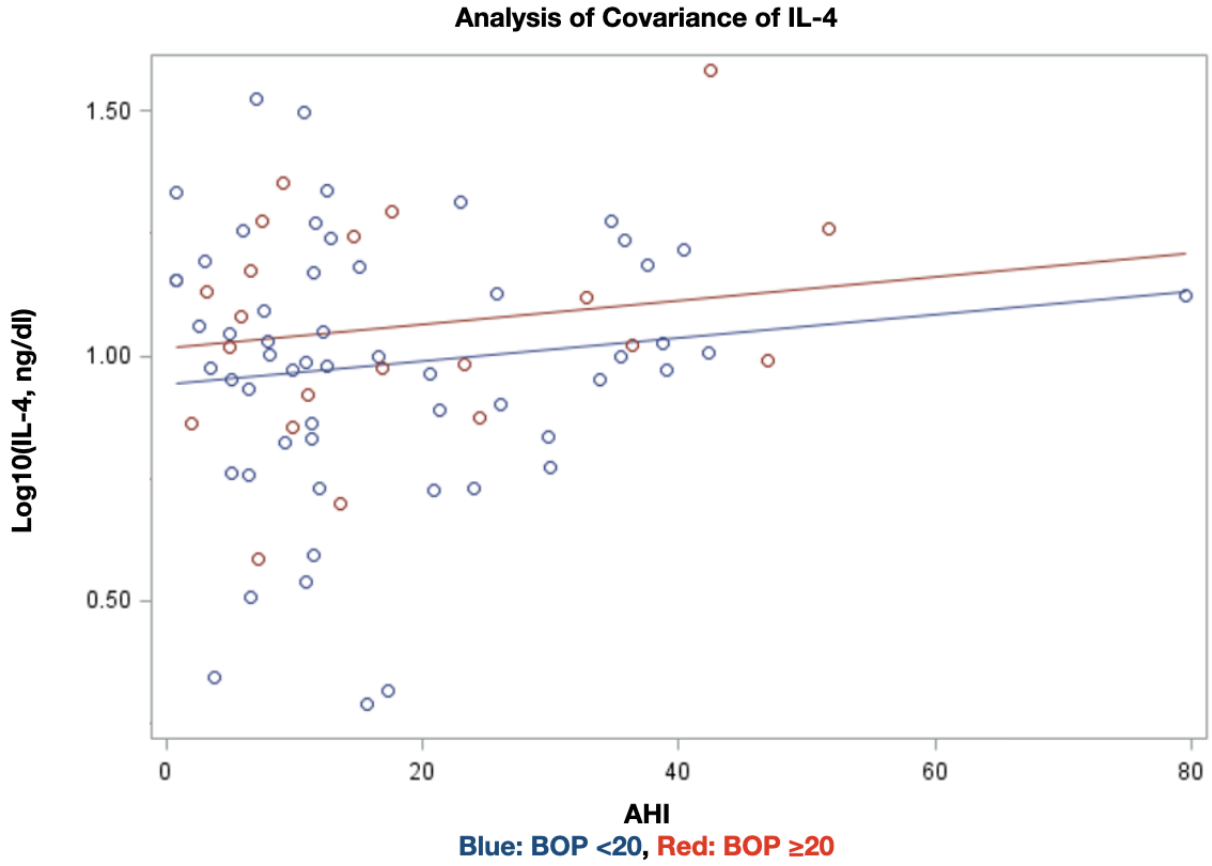

Analysis of Covariance of IL-4
Log10(IL-4, ng/dl)
1.50
1.00
0.50
0
20
40
60
80
AHI
Blue: BOP <20, Red: BOP ≥20

Figure 2.6:

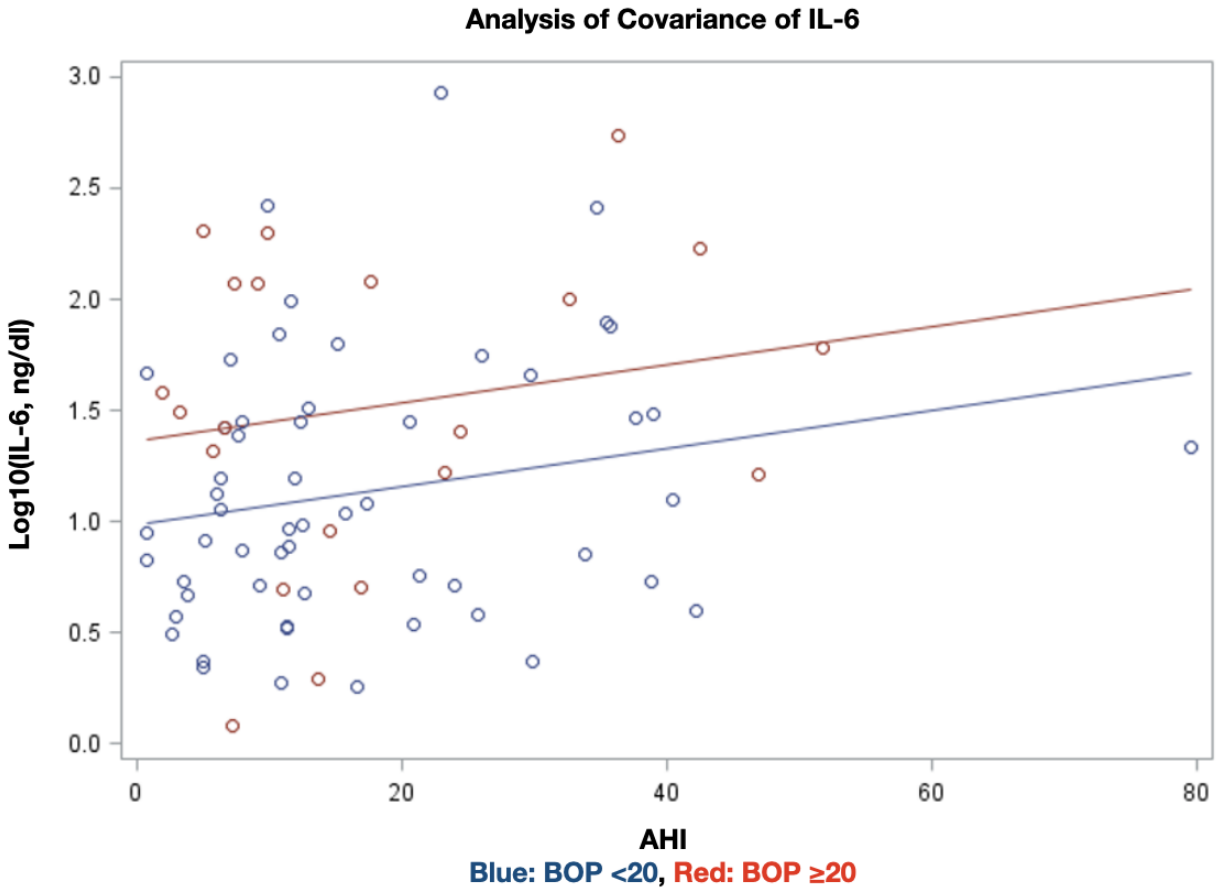

Analysis of Covariance of IL-6
Log10(IL-6, ng/dl)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
AHI
Blue: BOP <20, Red: BOP ≥20

Figure 2.7:

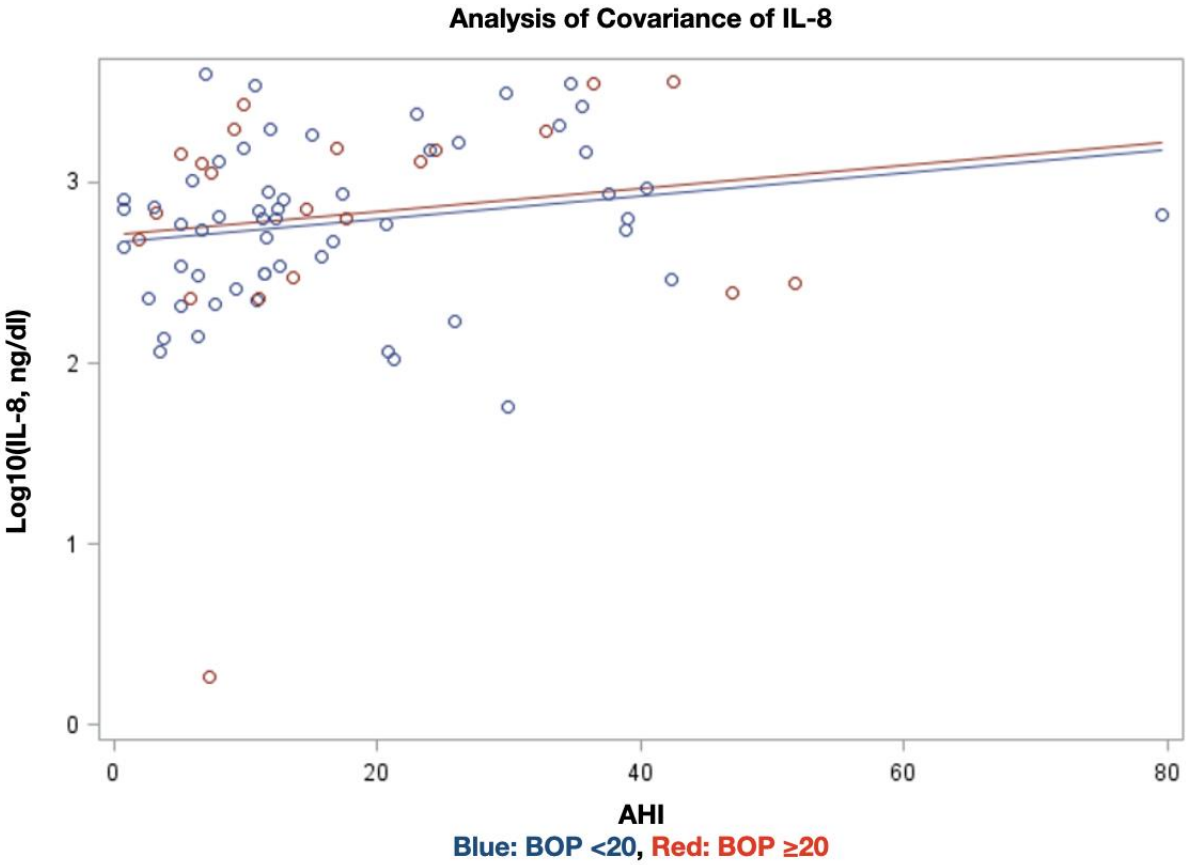

Analysis of Covariance of IL-8
Log10(IL-8, ng/dl)
0
1
2
3
0
20
40
60
80
AHI
Blue: BOP <20, Red: BOP ≥20

Figure 2.8:

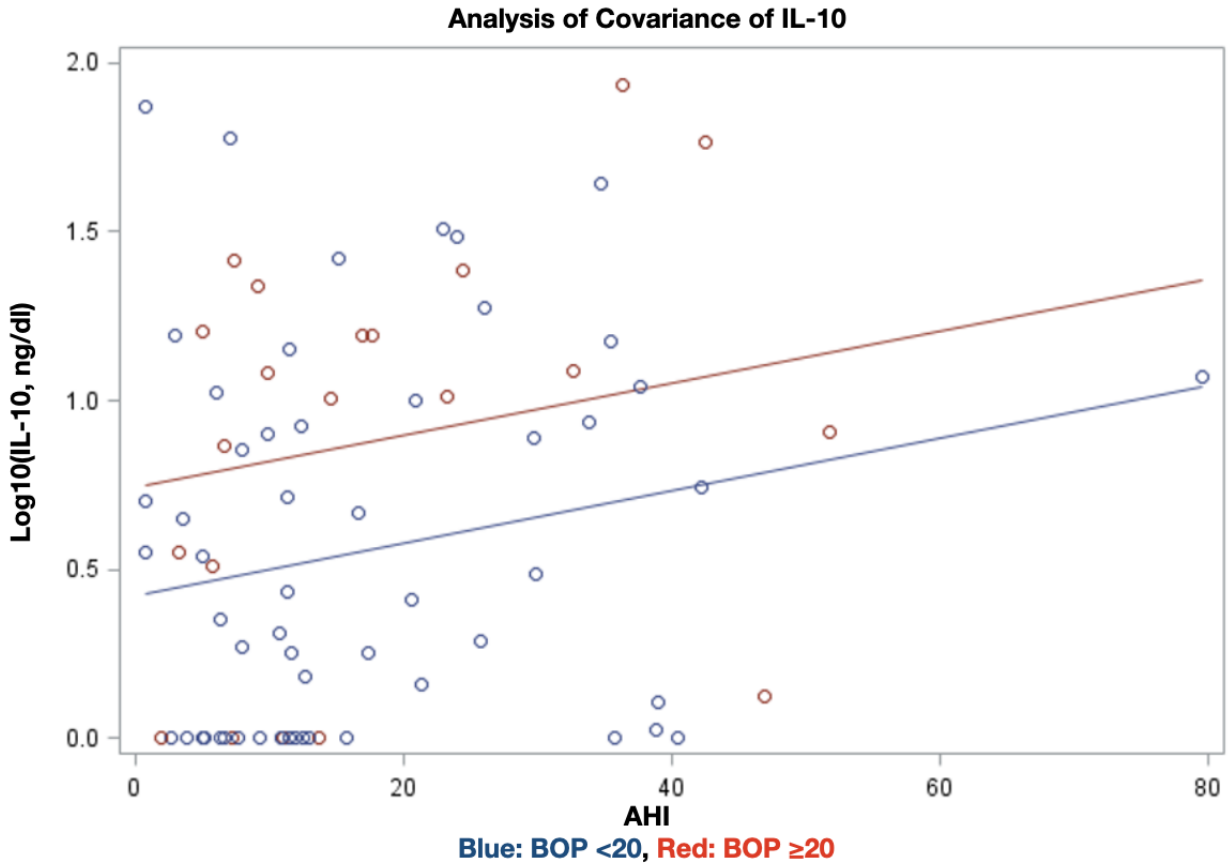

Analysis of Covariance of IL-10
Log10(IL-10, ng/dl)
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
AHI
Blue: BOP <20, Red: BOP ≥20

Figure 2.9:

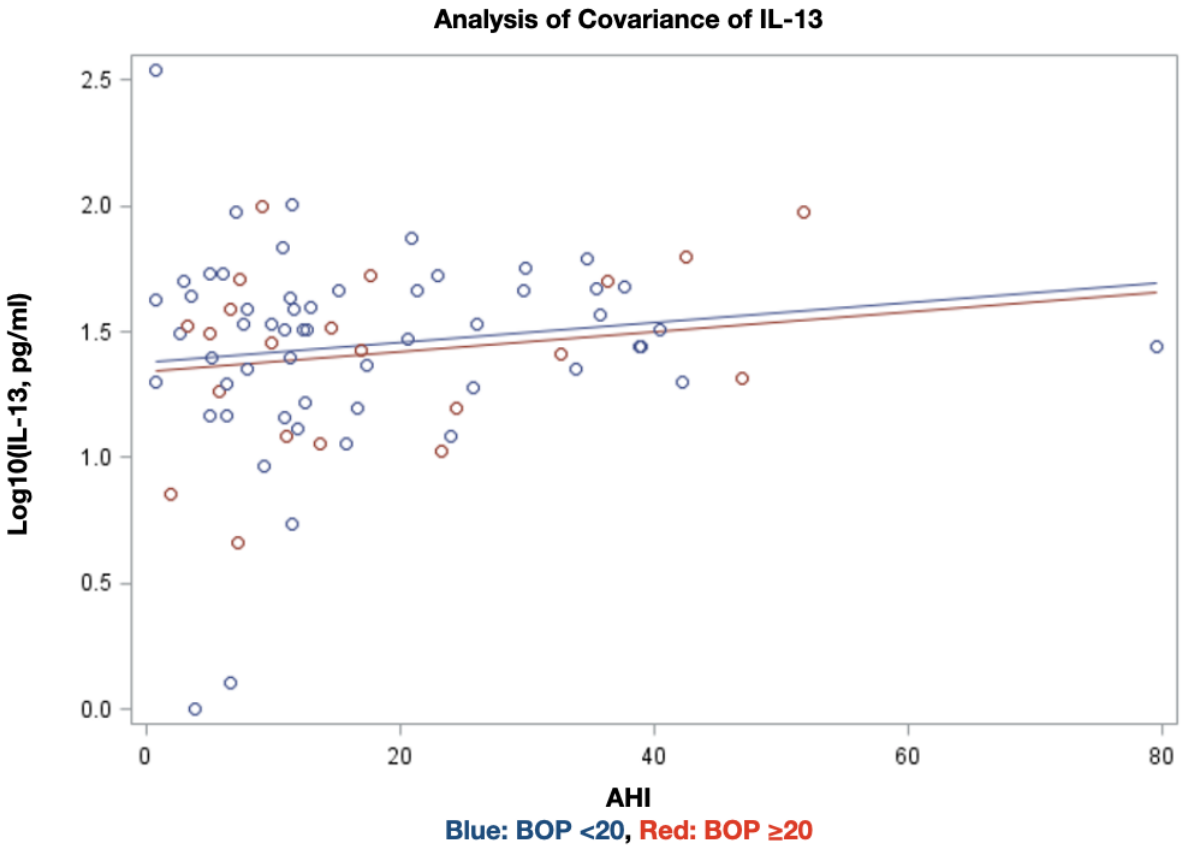

Analysis of Covariance of IL-13
Log10(IL-13, pg/ml)
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
AHI
Blue: BOP <20, Red: BOP ≥20

Figure 2.10:

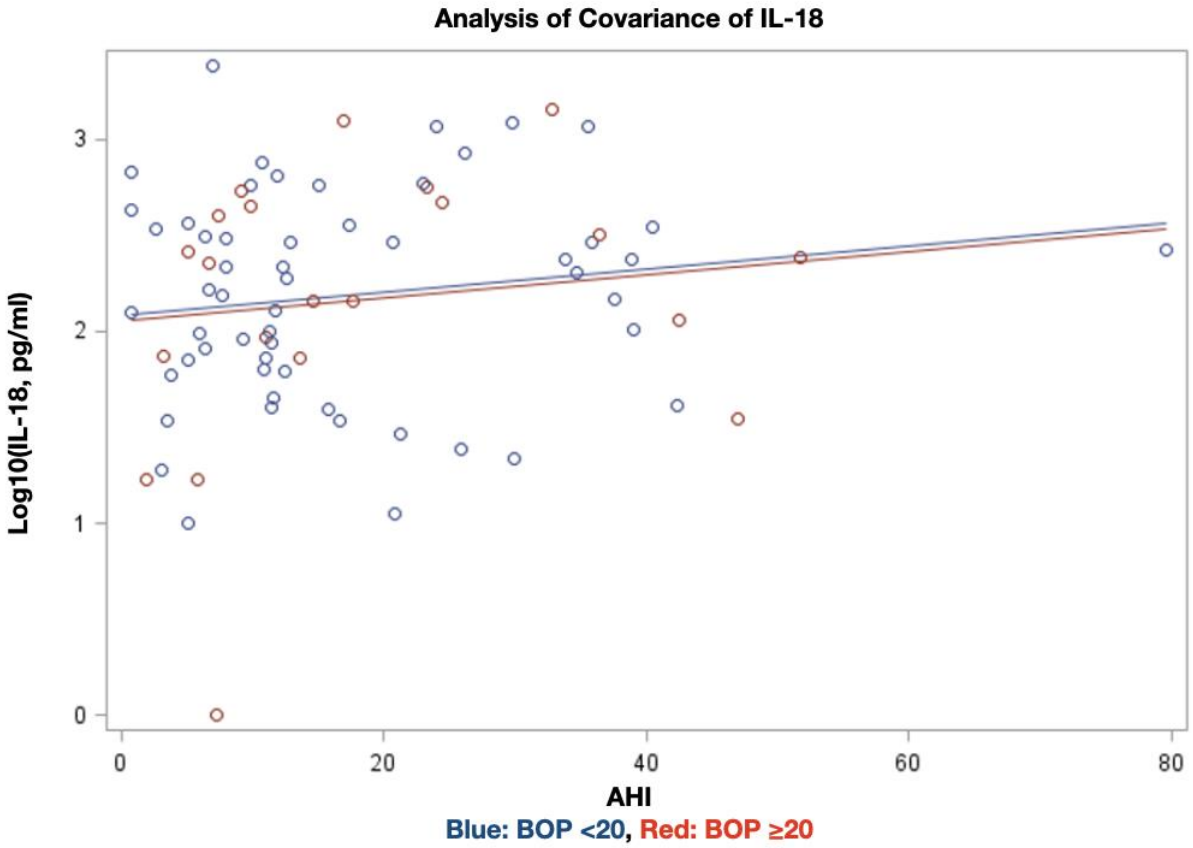

Analysis of Covariance of IL-18
Log10(IL-18, pg/ml)
3
2
1
0
0
20
40
60
80
AHI
Blue: BOP <20, Red: BOP ≥20

Figure 2.11:

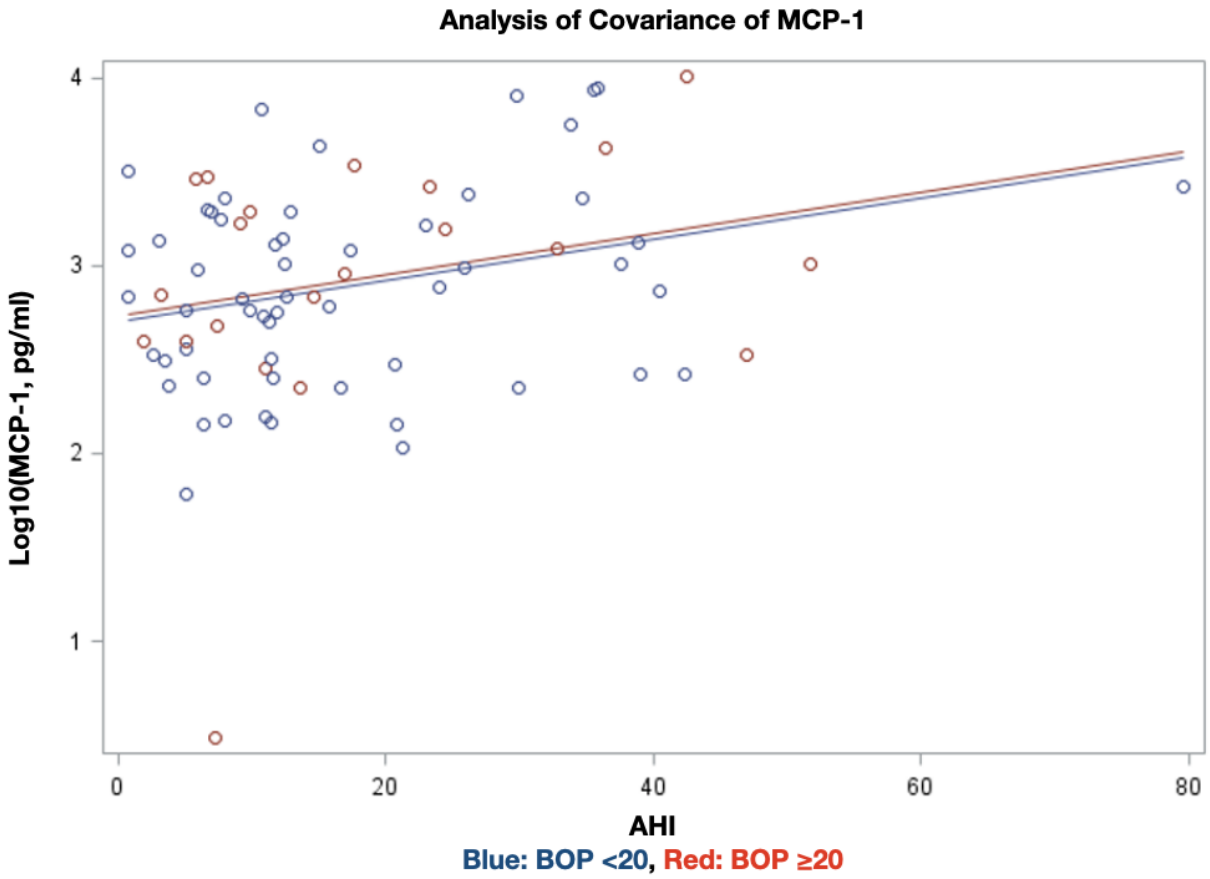

Analysis of Covariance of MCP-1
Log10(MCP-1, pg/ml)
4
3
2
1
0
20
40
60
80
AHI
Blue: BOP <20, Red: BOP ≥20

Figure 2.12:

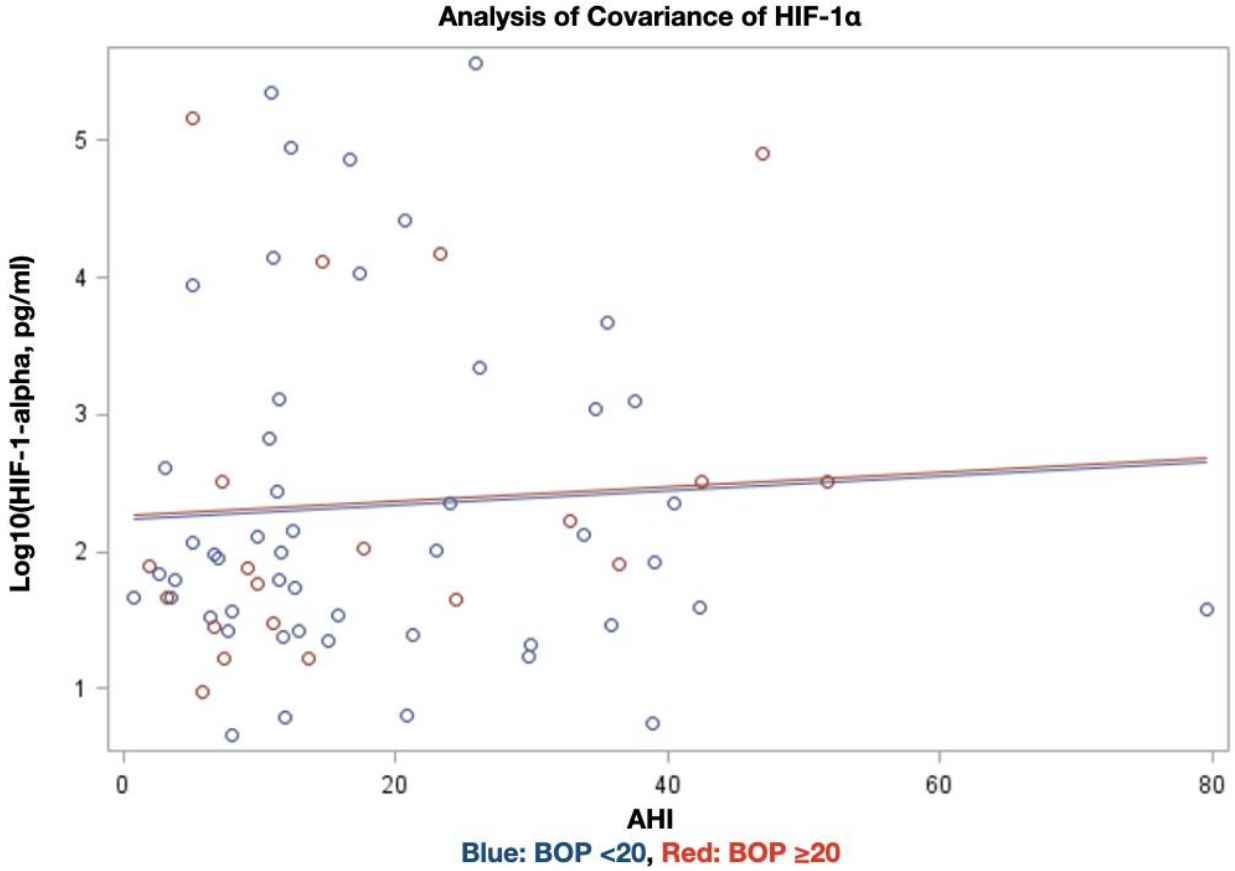

Analysis of Covariance of HIF-1α
Log10(HIF-1-alpha, pg/ml)
5
4
3
2
1
0
20
40
60
80
AHI
Blue: BOP <20, Red: BOP ≥20

Figure 2.13:

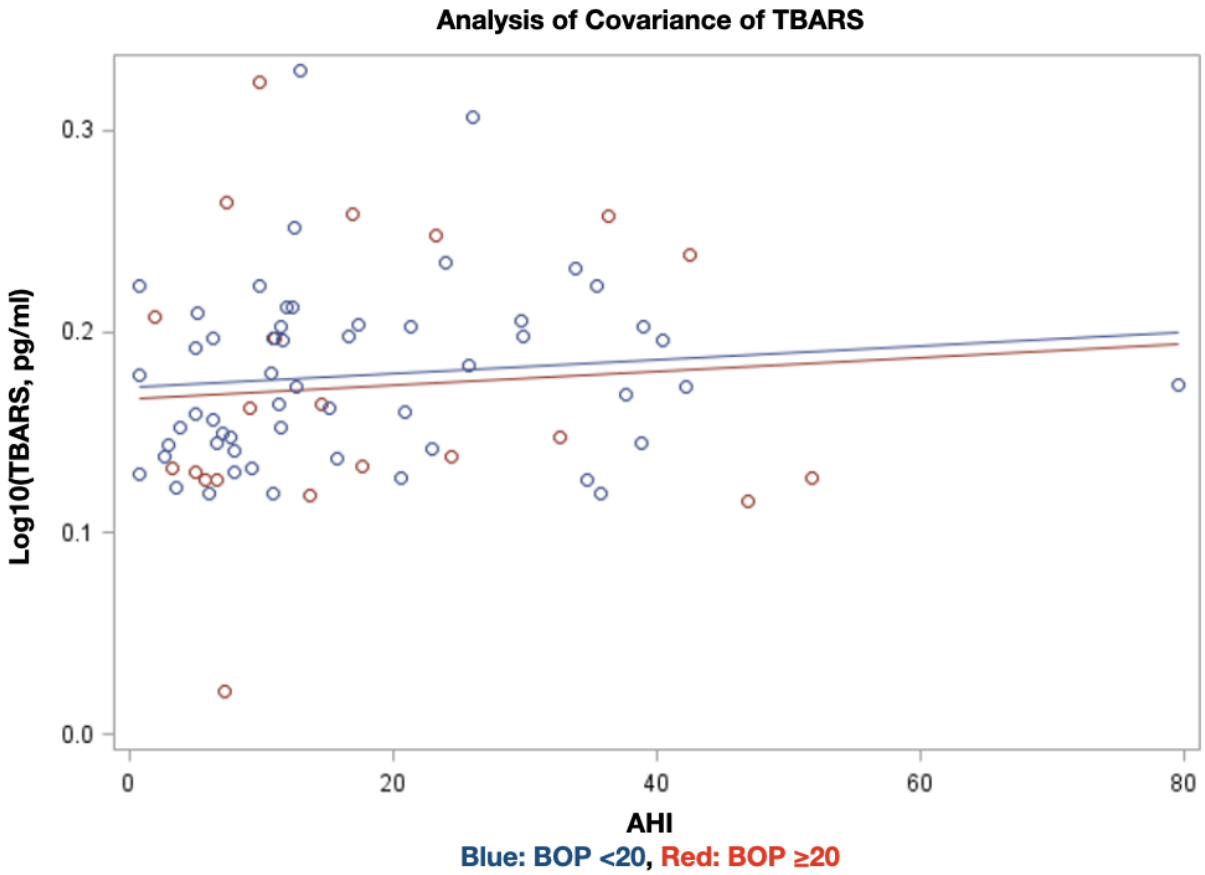
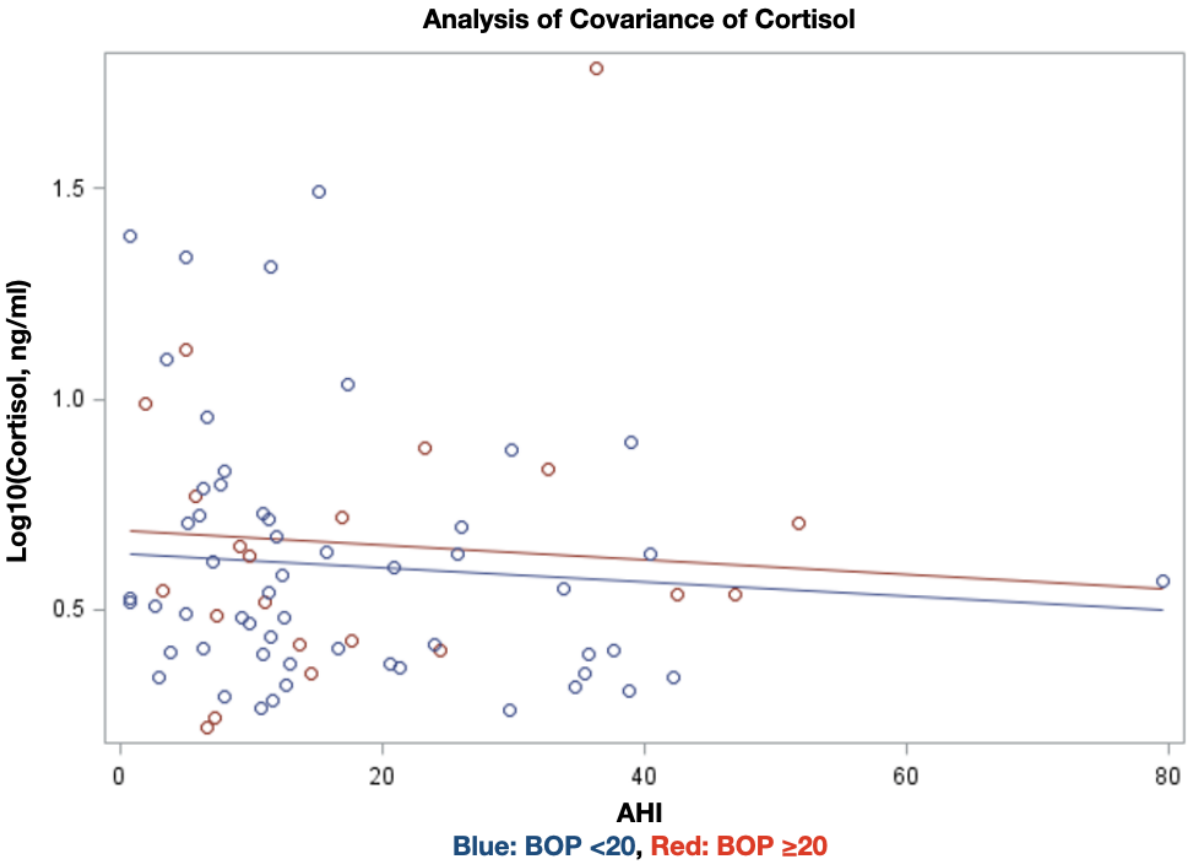

Analysis of Covariance of TBARS
Log10(TBARS, pg/ml)
0.3
0.2
0.1
0.0
0
20
40
60
80
AHI
Blue: BOP <20, Red: BOP ≥20

Figure 2.14:


Analysis of Covariance of Cortisol
Log10(Cortisol, ng/ml)
1.5
1.0
0.5
0
20
40
60
80
AHI
Blue: BOP <20, Red: BOP ≥20

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